บทบาทของตัวรับโปรเจสเตอโรนไอโซฟอร์มเอและบี ในมะเร็งปอดชนิดนิวโรเอนโดครายน์

นางสาวธีรนุท อัศวสุปรีชา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมีคลินิกและอณูทางการแพทย์ ภาควิชาเคมีคลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Role of Progesterone Receptor A and B Isoforms in Lung Neuroendocrine Tumors

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จุฬาลงกรณ์มหาวิทยาลัย
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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Clinical Biochemistry and Molecular
Medicine
Department of Clinical Chemistry
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2017

Thesis Title	Role of Progesterone Receptor A and B Isoforms in Lung Neuroendocrine Tumors
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Field of Study	Clinical Biochemistry and Molecular Medicine
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ธีรนุท อัศวสุปรีชา : บทบาทของตัวรับโปรเจสเตอโรนไอโซฟอร์มเอและบี ในมะเร็งปอดชนิดนิวโรเอนโดครายน์ (Role of Progesterone Receptor A and B Isoforms in Lung Neuroendocrine Tumors) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วิโรจน์ บุญรัตนกรกิจ, 88 หน้า.

ปรากฏการณ์ของผู้ป่วยที่เป็นโรคมะเร็งปอดชนิดนิวโรเอนโดครายน์มีการเพิ่มขึ้นในช่วงปีที่ผ่านมา ซึ่งสิ่งที่น่าสนใจคือ ผู้ป่วยเพศหญิงโรคมะเร็งปอดชนิดนิวโรเอนโดครายน์นี้มีอัตราการอยู่รอดที่ดีกว่าผู้ป่วยชาย คณะผู้วิจัยจึงสันนิษฐานว่าฮอร์โมนเพศ และ ตัวรับของฮอร์โมนเพศอาจส่งผลต่อการเจริญเติบโตของมะเร็งชนิดนี้ ข้อมูลปัจจุบันมีการรายงานว่าตัวรับโปรเจสเตอโรนมี ความเกี่ยวข้องกับอัตราการอยู่รอดของผู้ป่วยมะเร็งปอดชนิดนิวโรเอนโดครายน์ ซึ่งตัวรับโปรเจสเตอโรนนี้แบ่งออกเป็นสองไอโซ ฟอร์ม ซึ่งก็คือตัวรับโปรเจสเตอโรนไอโซฟอร์มเอและบี แต่อย่างไรก็ตามผลของการแสดงออกของตัวรับโปรเจสเตอโรนไอโซ ฟอร์มต่างๆต่อการเจริญเติบโตของมะเร็งและอัตราการอยู่รอดของผู้ป่วยยัง ไม่เป็นที่ทราบแน่ชัด จึงเป็นที่มาในการศึกษา ความสัมพันธ์ดังกล่าว คณะผู้วิจัยจึงสร้าง เซลล์โมเดลมะเร็งปอดนิวโรเอนโดครายน์ชนิด H727 ให้เกิดการแสดงออกของตัวรับโป รเจสเตอโรนไอโซฟอร์มเอและบี ซึ่งจากการทดสอบ เซลล์โมเดลมีการแสดงออกของโปรตีน กระบวนการถอดรหัสของยีน และ ที่ตั้งของโปรตีนตัวรับโปรเจสเตอโรน เฉกเช่นเดียวกับตัวรับโปรเจสเตอโรนภายในเซลล์มะเร็งเต้านม จากนั้นจึงทำการทดสอบดูผล ของการแสดงออกของตัวรับโปรเจสเตอโรนต่อคุณสมบัติทางชีวภาพของเซลล์ ซึ่งผลคือการแสดงออกของตัวรับโปรเจสเตอโรน โดยปราศจากโปรเจสเตอโรนยับยั้งการเจริญเติบโตของเซลล์ นอกจากนี้คณะผู้วิจัยได้สร้างแอนติบอดีที่จำเพาะต่อ เอนเทอมินัสของ ตัวรับโปรเจสเตอโรนไอโซฟอร์มบี (250H11) ซึ่งทดสอบความจำเพาะต่อการจับตัวรับโปรเจสเตอโรนไอโซฟอร์มบี แต่ไม่จับตัวรับ โปรเจสโตโรนไอโซฟอร์มเอโดยการทำอิมมูโนบลอทติ้งและแบบย้อมสารเรืองแสง จากนั้นจึงนำแอนติบอดีดังกล่าวมาย้อมชิ้น เนื้อโดยอิมมูโนพยาธิวิทยา ผู้ป่วย 198 รายถูกนำมาย้อมทางอิมมูโนพยาธิวิทยาโดยใช้แอนติบอดี 2 ชนิด คือ 1294 เพื่อย้อมตัวรับโป รเจสเตอโรนทั้งไอโซฟอร์มเอและบี และ 250H11 ย้อมตัวรับโปรเจสเตอโรนไอโซฟอร์มบีเท่านั้น จากผลการแสดงออกของตัวรับโป รเจสเตอโรนไอโซฟอร์มเอและบี พบว่า ผู้ป่วยที่มีการแสดงออกของตัวรับโปรเจสเตอโรนไอโซฟอร์มเอและบีพบว่ามีดัชนีชี้การ เจริญเติบโตของเซลล์ต่ำกว่าคนที่ไม่มีการแสดงออกของตัวรับโปรเจสเตอโรนไอโซฟอร์มเอและบี และ การมีตัวรับโปรเจสเตอโรน มักพบในผู้ป่วยมะเร็งปอดชนิดนิวโรเอนโดครายน์ประเภทที่ความรุนแรงของโรคน้อยอย่างมีนัยสำคัญ ยิ่งไปกว่านั้นสิ่งที่น่าสนใจคือ ผู้ป่วยที่มีการแสดงออกของตัวรับโปรเจสเตอโรนไอโซฟอร์มบีมีความสัมพันธ์กับการอยู่รอดปลอดโรคที่ยาวนานขึ้น ซึ่งนอกจากนี้ คณะผู้วิจัยได้มีการใช้แอนติบอดีที่มีความจำเพาะต่อตัวรับโปรเจสเตอโรนนี้กับการย้อมทางอิมมูโนพยาธิวิทยากับชิ้นเนื้อปอดปกติ ของตัวอ่อนและผู้ใหญ่ ซึ่งพบว่ามีการแสดงออกของตัวรับโปรเจสเตอโรนในเซลล์ปอดชนิดนิวโรเอนโดครายน์ในปอดของตัวอ่อน ในครรภ์และผู้ใหญ่ที่ปกติ ซึ่งสันนิษฐานว่าตัวรับโปรเจสเตอโรนอาจมีหน้าที่เกี่ยวกับการพัฒนาของเซลล์นิวโรเอนโดครายน์ จาก ข้อมูลที่ได้มาบ่งชี้ว่าการแสดงออกของตัวรับโปรเจสเตอโรนไอโซฟอร์มบีมีความเกี่ยวข้องกับมะเร็งปอดชนิดนิวโรเอนโดครายน์ที่ มีความรุนแรงของโรคและการเจริญเติบโตของเซลล์น้อยกว่า และมีความสัมพันธ์กับการมีการอยู่รอดปลอดโรคที่ยาวนานขึ้น ซึ่งการ เข้าใจกลไกทางโมเลกุลของสัญญาณของตัวรับโปรเจสเตอโรน หรือตัวรับโปรเจสเตอโรนไอโซฟอร์มต่างๆในมะเร็งปอดชนิดนิวโร เอนโดครายน์ อาจช่วยในการพัฒนาการรักษาสำหรับผู้ป่วยมะเร็งปอดชนิดนิวโรเอนโดครายน์ได้ในอนาคต

ภาควิชา เคมีคลินิก	ลายมือชื่อนิสิต	------------------------------------
สาขาวิชา ชีวเคมีคลินิกและอณูทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	------------------------------------
ปีการศึกษา 2560		

5876654137 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE

KEYWORDS: PROGESTERONE RECEPTOR ISOFROMS A AND B / LUNG NEUROENDOCRINE TUMORS / PRB-SPECIFIC ANTIBODY

TEERANUT ASAVASUPREECHAR: Role of Progesterone Receptor A and B Isoforms in Lung Neuroendocrine Tumors. ADVISOR: ASST. PROF.VIROJ BOONYARATANAKORNKIT, Ph.D., 88 pp.

Lung neuroendocrine tumors (NETs) incidences are increasing in recent years. Women with lung NETs have significantly better survival rates as compared to men suggesting the involvement of sex steroids and their receptors in the progression of lung NETs. Recent data suggested that progesterone receptor (PR) may play a role in the survival of lung NET patients. PR exists as two major isoforms, PRA and PRB. How expression of PR isoforms affects proliferation of lung NETs and patient's survival is not known. To determine the role of PR isoforms in lung NETs, we constructed H727 lung NET cell models expressing PRB or PRA. PR expressions, transcriptional activities and localization in these cell models were similar to endogenous PR in breast cancer cells. PR expression inhibited H727 cell proliferation in the absence of progestin. A monoclonal antibody specific to the N-terminus unique to PRB (250H11 mAb) was developed to specifically detect PRB, but not PRA, by immunoblots and immunostaining. Immunohistochemistry staining with 250H11 and 1294 mAb, detecting PRB and PRB&PRA, respectively, were performed on 198 cases of lung NETs tissue sections. Comparing with PR negative cases, PR-positive lung NETs showed significantly lower mitotic index and were found mostly in lower grade lung NETs. Interestingly, PRB expressions in lung NETs were associated with longer disease-free survival. Using these PR specific antibodies, we showed PR expression in pulmonary neuroendocrine cells of normal fetal and adult lung suggesting a role of PR in the development of neuroendocrine cells. Together, these data demonstrate that PRB expression is associated more differentiated less proliferative tumors and longer disease-free survival of patients with lung NETs. Better understanding of molecular mechanism of how PR or PR isoform signals in lung NET cells may help us to develop novel therapeutic strategies that will be beneficial for lung NET patients in the future.

Department:	Clinical Chemistry	Student's Signature ....
Field of Study:	Clinical Biochemistry and Molecular Medicine	Advisor's Signature ....
Academic Year:	2017	

ACKNOWLEDGEMENTS

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Meaning of symbols and abbreviations

Symbols and abbreviations	Meaning
%	Per cent
/	Per
α	Alpha
β	Beta
μ	Micro
μl	Microlitre
μg	Microgram
°C	Degree Celsius
AF-1	Tramscription activation function 1
AF-2	Tramscription activation function 2
AR	Androgen receptors
APS	Ammonium persulfate
Ab	Antibody
Bp	Base pairs
BSA	Bovine serum albumin
CO_2	Carbon dioxide
C-terminal	Carboxyl-terminus
CgA	Chromogranin A
rtTA	Reverse tetracycline-controlled transactivator
	RPMI
RPMI	Roswell Park Memorial Institute medium

SCLC	Small cell lung cancer
SH2 domain	SRC Homology 2 domain
SH3 domain	SRC Homology 3 domain
Shc	Src homology domain containing
SYN	Synaptophysin
SRE	Steroid response element
SRC	Steroid receptor coactivator
SDS	Sodium dodecyl sulfate
Tyr	Tyrosine kinase
TKI	Tyrosine kinase inhibitors
TRE	Tetracyline-responsive promoter element
T47D	Human ductal breast epithelial tumor cell line
TEMED	Tetramethylethylenediamine
kDa	Kilo Dalton
Kb	Kilo base
LBD	Ligand binding domain
M	Molar
Ml	Millilitre
Mg	Milligram
MAPK	Mitogen activated protein kinase
MTT	3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
mRNA	Messenger RNA

NSCLC	Non-small cell lung cancer
NR	Nuclear receptors
NaF	Sodium fluoride
Na_3VO_4	Sodium orthovanadate
nM	Nanomolar
OD	Optical density
P	P-value
pH	Potential of Hydrogen ion
PI3K/AKT	Phosphatidylinositol-3-kinase/AKT
PR	Progesterone receptor
PIs	Proteinase inhibitor cocktail
PBS	Phosphate Buffered Saline
Penstrep	Penicillin Streptomycin
Rpm	Revolutions per minute
Dox	Doxycyline
DNA	Deoxyribonecleic acid
DBD	DNA binding domain
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modification of Eagle's medium
DCC	Charcoal Stripped Fetal Bovine Serum
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
ER	Estrogen receptor

EDTA	Ethylenediaminetetraacetic acid
FDA	Food and drug Administration
FBS	Fetal bovine serum
g	Gram
g	G-force
HSP	Heat shock protein
HEK293T	Human Embryonic Kidney 293 cells

CHAPTER I

INTRODUCTION

Lung cancer is one of the most fatal cancers. Globally, lung cancer is the leading cancer in term of incidence and mortality (1). Base on neuroendocrine feature, lung cancer was divided into 2 types, non-small cell carcinomas (NSCLC) represent 75% of lung cancer, with the remaining 25% composed of lung neuroendocrine tumors (Lung NETs) (2). Lung NETs have gained attention in recent years (2, 3). The incidences of lung neuroendocrine tumors had an unexplained but substantial increase in the last 30 years (3). Currently, there are no optimal therapy for metastatic lung neuroendocrine carcinoid tumor (4). According to the 2015 World Health Organization (WHO) classification, there are four types of lung neuroendocrine tumors, including typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC), and small cell lung cancer (SCLC) (5). Large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancer (SCLC) are strongly related with smoking history but typical carcinoid (TC) and atypical carcinoid (AC) have no preference for smoking history (2, 3). Furthermore, there are evidences suggested that the 5-year observed survival of women and men in lung neuroendocrine tumor are quite different. The prognosis of lung neuroendocrine tumors for women are far better than those of men (6), suggesting that steroid hormones may play a role in the development or progression of lung neuroendocrine tumors. PR expression was also have been studied, several reports found PR expression in lung neuroendocrine tumors (7, 8). Previous study also suggests that PR positive cases tend to have shorter survival in SCLC and combined SCLC (8). PR exists as two isoforms, PRA and PRB. PRA lacks the first 164 amino acids at N-terminus of PRB. In breast cancer, over-expression of PR-A or PR-B in the mammary gland of transgenic mice results in abnormal development (9). A high PR-A: PR-B ratio in breast cancers is associated with a more aggressive phenotype, with resistance to endocrine therapy, with a poorer disease–free survival (10). In NSLC, PRB expression interfered with epidermal growth factor (EGF)-induced NSLC cell proliferation and activation of ERK1/2, in the absence of progestin (11). Roles PR isoform in the etiology and progression of lung neuroendocrine tumors are not known.

Therefore, in this study we aimed to examine how differential expression of PR isoforms affected lung neuroendocrine tumor cell proliferation and determine whether expression of PR isoforms was associated with clinical outcomes of lung neuroendocrine tumor's patients.

Objectives (Aims)

1.   To analyze effects of progesterone receptor isoform expressions on biological properties of H727 neuroendocrine tumor cells.

2.   To examine progesterone receptor and progesterone receptor isoform B expression in Lung neuroendocrine tumors tissues and correlates progesterone receptor isoforms expression with clinicopathological factors such as lung NETs types, mitotic index, tumor stages, metastasis, tumor sizes and 10-years survival rates.

3.   To examine progesterone receptor and progesterone receptor isoform B expression in pulmonary neuroendocrine cells of normal fetal and adult lung.

Research questions

Do progesterone receptor isoform A and B have differential biological effects in lung neuroendocrine tumor?

Hypothesis

Expression of progesterone receptor isoforms B reduces lung neuroendocrine tumor cell proliferation and correlates with better prognosis of lung neuroendocrine tumor patients.

Scope and Limitation of the Study

In this research, we determined whether expression of PR isoforms was associated with clinical outcomes of lung neuroendocrine tumor's patients. We used H727 lung neuroendocrine tumor cell line to construct H727-PRA and H727-PRB cell models. Those two cell models were induced to express PRA or PRB by addition of tetracycline. Western blotting, immunofluorescence and luciferase reporters analyses were used to validate PR expression, localization and transcriptional activities in these

cell models. Cell models' biological properties were examined by MTT assay to evaluate cells viability. Immunohistochemistry was used to determine PR and PRB expression in lung NETs patients' tissues. We then correlated PR and PRB expression data with clinicopathological factors and survival rates. Additional, we also examined PR and PRB expression in pulmonary neuroendocrine cells of normal fetal and adult lung.

Usefulness of the Study

The correlation between progesterone receptor isoform expression and its association with clinical outcomes such as sex, age, mitotic index, tumor stage, metastasis, tumor size and survival rates may help with the diagnosis and prognosis of lung neuroendocrine tumor patients. Furthermore, understanding the role of PR in lung neuroendocrine tumors can help us to develop innovative molecule targeting the progesterone receptor signaling for treatment of lung neuroendocrine tumors patients in the future.

CHAPTER II
LITERATURE REVIEW

Neuroendocrine Tumors

Neuroendocrine tumors (NETs) arise from neuroendocrine cells throughout the body. It was comprised of a heterogeneous group of malignancies. Neuroendocrine tumor patients are characterized by a relatively slow tumor growth requiring long term medical therapy, while some progress rapidly. Well-differentiated neuroendocrine tumors have low mitotic rates while poor-differentiated has high mitotic rates. Well-differentiated neuroendocrine tumors of all sites share similar basic morphology, coarsely granular "salt and pepper" chromatin, lack of prominent nucleoli, overall uniformity, organoid nest, trabeculae and rosettes (2). Poor-differentiated neuroendocrine usually indicated small cell and large cell carcinomas (2, 12). In immunohistochemistry, there are neuroendocrine markers which include synaptophysin, chomogranin A and CD56/NCAM which morphology features, mitotic index and neuroendocrine tumor markers can be used for diagnosis (13). Neuroendocrine tumors (NETs) are neoplasms most commonly in lung and gastrointestinal tract (14).

Gastro-intestinal and Pancreatic Neuroendocrine Tumors

In gastro-intestinal and pancreatic neuroendocrine tumor have diverse biological activities according to the primary tumor origin, type of neuroendocrine cell, and pathologic features (12, 13). The distribution patterns of NETs in the GI tract seem to be different between Eastern populations. It showed that rectum (48%) is the most frequent location of NETs in the GI tract of patients, followed by the stomach (15%), pancreas (9%), colon (8%), small intestine (8%), liver (7%), appendix (3%), and biliary tract (2%) (15). Neuroendocrine tumor grade in GI tract divided into NET (G) 1, NET G2, and NECs, based on mitotic counts and the Ki-67 proliferation index, regardless of tumor size, extent, or location, according to WHO classification 2010 (16). Neuroendocrine markers were also used for diagnosis. Synaptophysin is considered the most sensitive neuroendocrine marker, chromogranin A is the most specific and other neuroendocrine markers, such as CD56/NCAM1, Leu7, and

neuron-specific enolase, are not recommended because of their low specificity (13). Endoscopic resection, surgical resection and lymph node dissection are the treatment of choice (17, 18). However, usefulness of multi-drug chemotherapy remains to be evaluated in larger clinical studies (19, 20).

Lung Neuroendocrine Tumors (Lung NETs)

Origin: Pulmonary Neuroendocrine Cells

Lung NETs originate from pulmonary neuroendocrine cells (PNECs)(21) Pulmonary neuroendocrine cells (PNEC) represent in airway epithelial which occur less than 1% of total lung epithelial cell proliferation (22) (Fig1). PNEC especially presented in fetal and neonatal development more than in elderly (23, 24). Pulmonary neuroendocrine cells composed with amine and peptides-producing cells. (25, 26) PNEC secrete bombesin like peptides, leads to lung branching morphogenesis,(27) growth and maturation (28, 29).
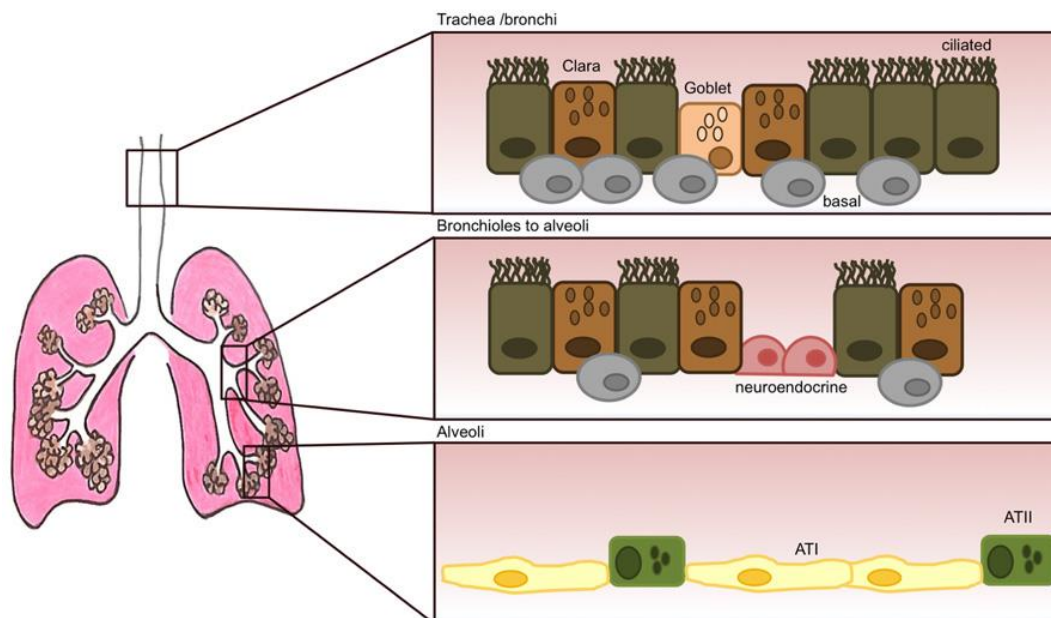
Figure 1 Diverse cell population in respiratory epithelium (30)

Classification, diagnosis, treatment and etiology of lung NETs

Lung NETs represent 25% of primary lung neoplasms, with the remaining 75% composed of non–small cell carcinoma (NSCLC) (2). In lung NETs, the incidences had an unexplained but substantial increase in the last 30 years rising from 0.3 cases per 100,000 in 1973 to 1.35 cases per 100,000 in 2004. There are 4 types of lung neuroendocrine tumor according to 2015 World Health Organization (WHO) classification, there are typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC), and small cell lung cancer (SCLC). In WHO classification 2015, all types of lung NET were combined into one category to facilitate differential diagnosis (31) (Fig 2), whereas carcinoids were grouped separately from LCNECs and SCLCs in the 2004 WHO classification (32). Morphologic criteria for distinguishing lung NETs are morphological feature, presence or absence of necrosis and the number of mitoses (Fig2). The detection of immunohistochemical markers, chromogranin A, synaptophysin, and CD56 were also used to confirm a diagnosis of lung NET, (31). Moreover, The updated 2015 WHO classification for lung NETs also guided to use the Ki-67 cell proliferation labeling index to distinguish between high-grade lung NETs (>40%) and carcinoids (<20%) (31).
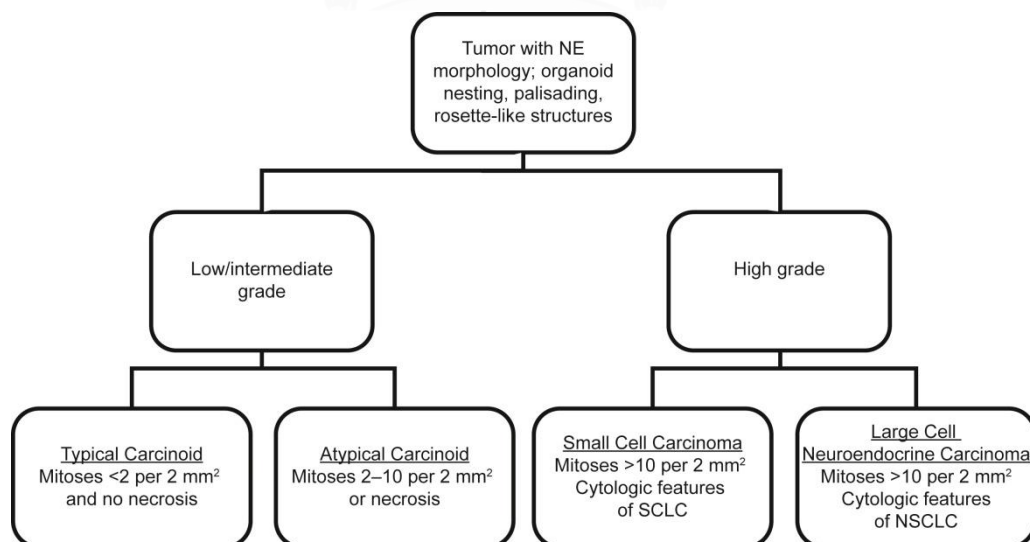
Figure 2 Morphologic criteria for distinguishing lung NET (5)

Recently treatment of lung NETs, only SCLC has standard treatment for metastasis cancer. SCLC patients usually have an excellent response to chemotherapy with or without initial radiotherapy (33). There is no standard treatment of pulmonary LCNEC but surgery could be a primary optional for operable patients. Inoperable disease treatment could use chemotherapy or radiotherapy (34). TC and AC metastatic disease treatment also not well studied but could use chemotherapy and radio therapy or surgery (35, 36). For localized disease, surgical resection is the treatment of choice in patients of TC and AC (37).

For large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancer (SCLC) are strongly relate with smoking history (38, 39). But carcinoid tumors, which are typical carcinoid (TC) and atypical carcinoid (AC) have no preference for smoking history (40). Furthermore there are evidences shows that the 5-year observed survival of women and men in lung neuroendocrine tumor are different, the prognosis of neuroendocrine tumors for women are better than men (41). Sex steroid hormone and their receptors could play an important role in lung NET.

Steroid hormones and steroid hormone receptors

Steroid hormones

Steroid hormones are synthesized in various organ including gonads, adrenal glands, and the feto-placental unit. Precursor of all steroid hormones is cholesterol. Cholesterol converted to pregnenolone then converted to glucocorticoids, or mineralocorticoids in steroidogenic pathway (Fig3). In the sex hormone pathway, pregnenolone is first converted to progesterone, and then synthesize androgens and estrogens respectively (42). Sex steroid hormones act via their receptors such as estrogen via estrogen receptor (ER) α or ERβ, progesterone via progesterone receptor (PR)-A or PR-B (43, 44)
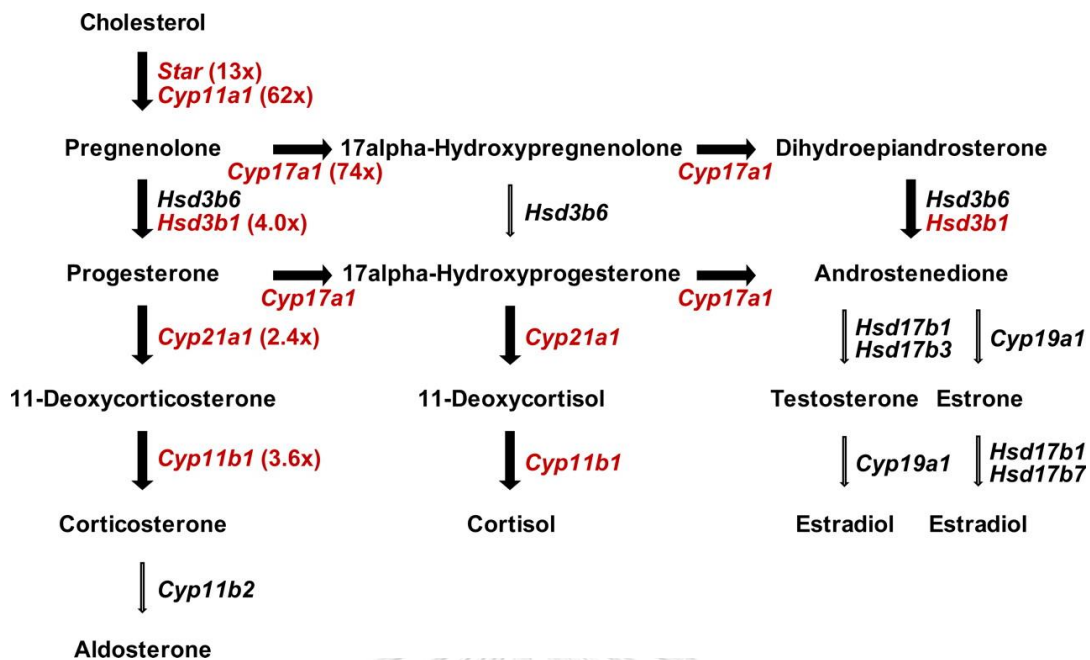
Figure3 Steroid hormone synthesis (45)

Steroid hormones receptors

Steroid hormone receptors were subset of nuclear receptor superfamily which located both in nucleus and cytoplasm. Nuclear receptors grouped into four classes according to their ligand binding, DNA binding, and dimerization properties included steroid receptors, RXR heterodimers, homodimeric orphan receptors and monomeric orphan receptors. Structure of steroid hormone receptor consists of 3 domains which are DNA binding domain, ligand binding domain and transcription activation function which has at least two transcription activation function, AF-1 locate in N-terminal and AF-2 is in ligand binding domain (46) (Fig4).

Nuclear Receptor Structure

Figure4 Nuclear receptor structure (47)

Steroid hormone receptors consist of 2 signaling pathway, classical or genomic signaling pathway and extranuclear or non-genomic signaling pathway. In genomic signaling pathway, steroid hormone receptors were form homodimer and bind to an inverted repeats of DNA (48, 49). At first, before hormone interacted with the receptor, receptor was bind heat shock protein in cytoplasm. After hormone interacted with the receptor, receptor formed homodimer then translocate to the nucleus to bind to its hormone response elements which effected transcriptional regulation (49). Non-genomic signaling pathway occurred in cytoplasm or membrane. Nuclear receptor interacted with cytoplasmic or cell membrane signaling molecules or adaptor such as c-src tyrosine kinase (Src) which induced phosphorylation of mitogen-activated protein kinase (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascade (50). Steroid hormone receptors that related with lung NETs are estrogen receptor and progesterone receptor (7, 8) (Fig5).
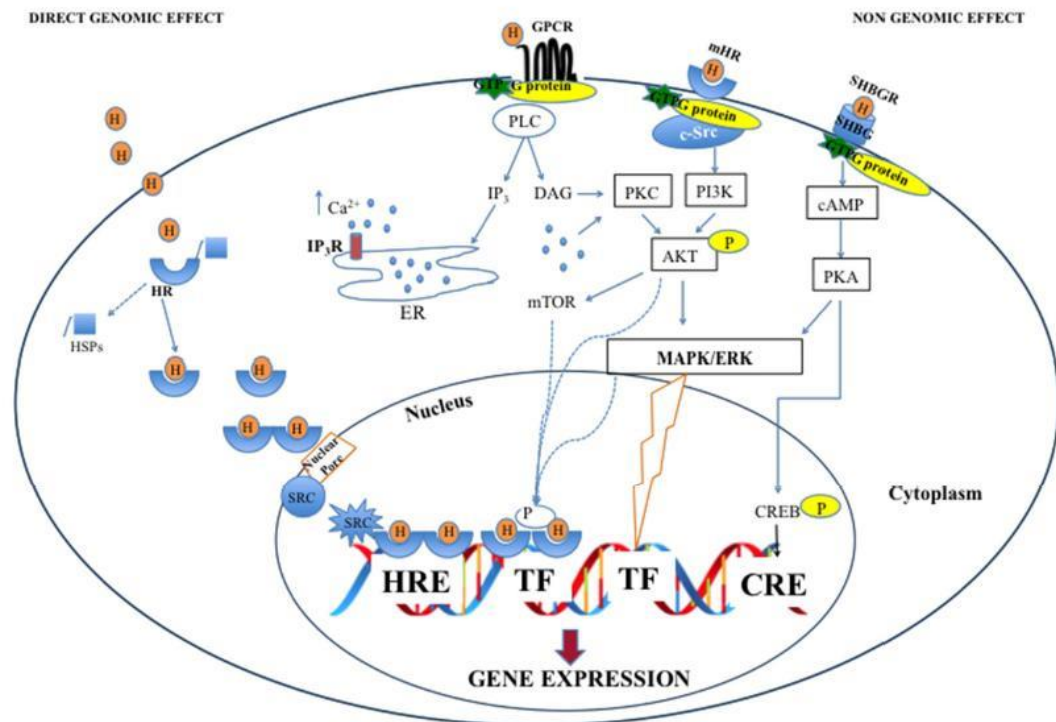
Figure 5 genomic and non-genomic of nuclear receptor pathways (51)

Association of Steroid hormone receptors and prognosis of cancers

Estrogen receptors (ER)

ER encoded by two genes: alpha and beta (ERα and ERβ) (52). There is several evidences showed association between estrogen receptors and breast cancer (53, 54). ER isoforms also have evidences of association between breast cancers. Immunohistochemical (IHC) examination of ERα status is the standard for pathological evaluation which used to guide anti-estrogen therapy (55). However, ERβ, its clinical significance remains unclear but some report showed that ERβ expression improved overall and disease free survival (56). Lung also associated with estrogen and estrogen receptor. In the normal lung tissue of humans and rodents, ER plays important physiological functions to promote proliferation and are required for alveolar formation during the development and maintenance of the pulmonary diffusion capacity (57). Estrogen has been shown to stimulate proliferation of NSCLCs far better than surrounding non-neoplastic lung fibroblasts (58). Inhibition of aromatase, a key enzyme for estrogen synthesis, suppresses lung tumor growth (58,

59). The presence of ERα in the absence of ERβ in NSCLC tumors was found to be associated with poor prognosis (60). In lung neuroendocrine tumor, recent study demonstrated ER isoforms expression in lung neuroendocrine tumor. Results ERβ leads to better prognostic for SCLC patients and especially SCLC male patients (7).

Progesterone receptors (PR)

PR are transcription factors that consist of N-terminal region in upstream that contains activation function (AF), DNA binding domain (DBD), a downstream hinge region and C-terminal ligand binding domain (LBD) (61-63). Progesterone receptors have two isoforms which are progesterone receptor isoform A and B (PRA and PRB). Form A lack the first 164 amino acids at N-terminus when compare with form B. Both of progesterone receptor isoforms A and B contains two activation function (AF1 and AF2) while PRB contains three activation functions (AF3, AF1, and AF2) which AF3 is in the B-upstream segment (BUS), the far N-terminal 164 amino acids distinct from PRA (61, 64) (Fig6). In general, PRB are stronger transactivators than PRA (64). On the other hand, several evidences showed that PRA can dominantly inhibit PRB (65, 66). PRA and PRB have different subcellular distributions in the absence of ligand, PRA primarily located in nucleus whereas PRB presents in both nucleus and cytoplasm. In the presence of ligand, PRA and PRB give complete nuclear translocation (67).
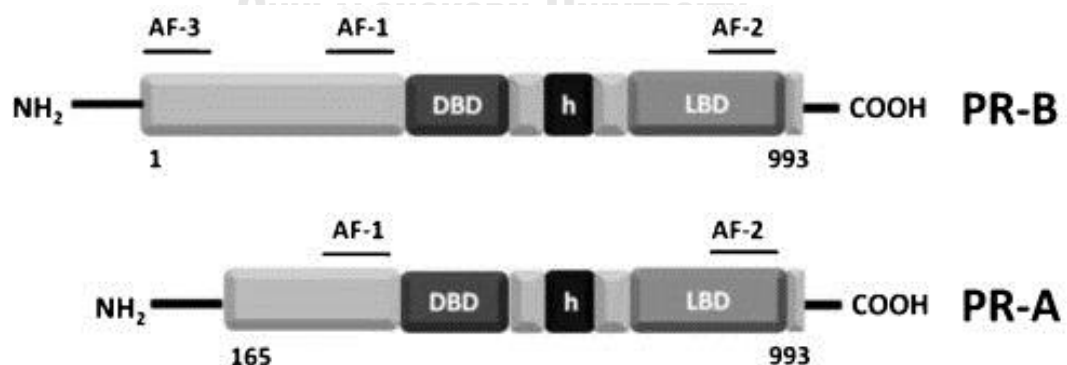
Figure 6 Structure of Progesterone receptor isoforms A and B (68)

Based on phenotypes of PR isoform selective knockout mice, PRB is more important for the proliferative responses to P4 in the mammary epithelium, while ovarian and uterine development and function rely primarily on PRA (69)

The relation of Progesterone receptors (PRs) and breast cancer shows that progesterone increase proliferation and number of progenitor cells in normal human breast which this may increase the risks of breast cancer (70). Moreover, when give RU486, which is anti-progestin, will reduce breast cell proliferation (71). However, the physical effects of progesterone are mediated by the progesterone receptor (72). Therefore, the different expression of PR isoforms may play a role with the clinical outcome for cancer. An increase in the relative levels of one PR isoform over the other (most frequently a predominance of PRA over PRB) has been observed in a high fraction of atypical hyperplasia, DCIS, and invasive breast cancer (73, 74). Forced overexpression of PRA relative to PRB in breast cancer cell lines leads to altered effects of progestin on cytoskeleton and cell mobility (75), and over-expression of PRA or PRB in the mammary gland of transgenic mice results in abnormal development (9). A high PRA: PRB ratio in breast cancers is associated with a more aggressive phenotype, with resistance to endocrine therapy, with a poorer disease free survival (10). Thus, altered progesterone signaling through changes in the normal ratios of the two PR isoforms could contribute to the etiology of breast cancer (76).

PR are also a prognostic factor in non-small cell lung cancer, progesterone inhibit growth of PR positive non-small cell lung cancer cells (77). Non-small cell lung cancer patients who have progesterone receptors expression often reported to correlate with better prognosis (77, 78). In NSLC, PRB expression interfered with EGF-induced NSLC cell proliferation and activation of ERK1/2, in the absence of progestin (11).

PR positive in gastro-intestinal neuroendocrine tumor patients were associated with lower stages disease and favorable prognosis (79). PR expression in pancreatic neuroendocrine tumor (pNET) patients correlated with absence of metastasis and better survival rates (80, 81). PR isoforms were detected and found that PRA could play an inhibitory role in the cell proliferation (82).

On the other hand, there is evidence of PR expression in lung neuroendocrine tumors (7, 8) and normal lung (83). In normal lung, there are evidences that progesterone and progesterone receptor are important for lung development and respiratory functions. In fetal, human lung has high progesterone concentration (84).

In mice ER and PR mRNA expression were highest in prenatal lung and significantly decrease after postnatal and adult lung (85) and in newborn piglet, antagonist of estrogen and progesterone receptor leads to impaired alveolarization and amiloride-sensitive alveolar fluid clearance (AFC) (83) This mean that both estrogen and progesterone are important for lung development and maturation. Furthermore, administration of progesterone combination with estrogen increase level of mRNA encoding rENaC subunit which plays a critical role in reabsorb alveolar fluid in immature rats (86). In adult, progesterone also has an effect to a respiratory function. In adult cat both male and female, progesterone acts as respiratory facilitation (87), in male rat, when treat with estrogen and progesterone leads to increased tidal volume, decrease arterial P_{CO_2} and enhanced the ventilator response to CO_2 inhalation (88). Moreover, Progesterone effects in upper airway dilator muscle activity(89) and PR regulated by progesterone in airway smooth muscle (ASM) and airway smooth muscle cells (ASMC) (90).

However, the relation between PR isoforms and clinical outcomes of the patients in lung neuroendocrine tumors is unknown. We hypothesize that the expression of PRB reduces lung neuroendocrine cancer cell proliferation and correlates with better prognosis in lung neuroendocrine tumor patients. In this study we examine how differential expression of PR isoforms affected lung neuroendocrine tumor cell proliferation and associated with clinical outcome of lung NET patients.

PRB (250H11) specific monoclonal antibody

In this study we used PR (1294) specific antibody and PRB (250H11) specific antibody to examine how differential expression of PR isoforms affected lung neuroendocrine tumor cell proliferation and associated with clinical outcome of lung NET patients. PR (PRA and PRB)-specific (1294) monoclonal antibody is a well-known antibody which detects an epitope at the N-terminus common to both isoforms A and B (91). Most antibodies detecting PR expression detect both PR isoforms, making it difficult to associate PR isoform expression with clinical outcomes in patient samples. To better understand the role of PRB and PRA in lung NET cells, we generated a new PRB specific mouse monoclonal antibody (250H11 mAb). PRB-

specific (250H11) monoclonal antibody detects an epitope at the N-terminus unique to PRB which we characterized. In previous study, PRB-specific antibodies; hPRa6 and Ab-6 were used. Previous PRB-specific antibodies were validated by immunohistochemistry. However, lack is known in western blot in Ab-6 PRB specific antibody. (92). Previous PRB antibody (hPRa6) was done by western blot but has less specificity. Using immunoprecipitation before western blot showed specificity of hPRa6 but using whole lysates for western blot failed to show specificity, PRA was detected (93). PR (1294) and PRB (250H11) specific monoclonal antibody were produced by Dean Edward, Baylor College of Medicine. PR (1294) and PRB (250H11) specific antibody were characterized in T47D cell line by western blot. Result showed that PR (1294) specific monoclonal detect both PRA and PRB expression in T47D cell line. However, PRB (250H11) specific monoclonal antibody could detect only PRB but not PRA (Fig7).
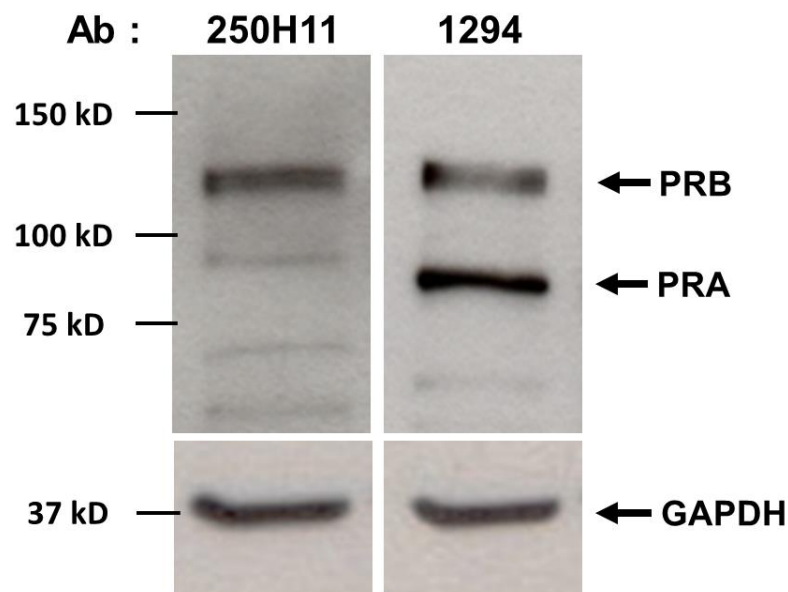
Figure 7 T47D expressing PRA and PRB were lysed and analyzed by PRB-specific mouse monoclonal antibody, (250H11 mAb) and PR-specific mouse monoclonal antibody, (1294 mAb) by Western blot analysis. (Prangwan Pateetin, Clinical Biochemistry and Molecular Medicine program, Chulalongkorn University)

CHAPTER III

MATERIALS AND METHODS

Conceptual framework

> **Lung neuroendocrine tumor (lung NET) patients have an unexplained but substantial increase in last 30 years. Evidence suggest that steroid hormone and steroid hormone receptors especially progesterone and progesterone receptors could play and important role with lung NET. However, roles of PR isoforms in lung NET patients still unknown.**

> **Hypothesis: Expression of PR B reduces lung neuroendocrine cancer cell proliferation and correlates with better prognosis in lung neuroendocrine tumor patients.**

> **Construct Tet-inducible PRA and PRB expression in H727 lung NETs cells.**
> Produce lentiviral particles to transduce PRA or PRB into H727 lung NETs cell line

> **Validate Tet-inducible PRA and PRB in H727 lung NETs cell model.**
> Detect the expression of PRA and PRB expression in H727 cells by Western blots
> Detect the localization of PRA and PRB expression in H727 cells and characterize PRB-specific antibody by Immunofluorescence
> Detect the transcriptional activity of PRA and PRB expression in H727 cells by luciferase assay

> **Characterize the biological properties of Tetracycline-inducible PRA and PRB expression in H727 lung neuroendocrine cancer cells with and without progesterone**
> Test cell proliferation by MTT assay

> **Examine progesterone receptor isoforms in lung NET patients by immunohistochemistry**
> Test Progesterone receptor isoforms (PR and PRB)

> **Correlate progesterone receptor isoforms expression with clinical outcomes such as tumor stages, metastasis, tumor size and 5-years survival rates and types of lung NETs**

> **Examine progesterone receptor isoforms in pulmonary neuroendocrine cells in normal lung by double immunohistochemistry**
> Test Progesterone receptor isoforms (PR and PRB) in pulmonary neuroendocrine cells(SYN)

Materials

Plasmids for lentiviral production

1. pHAGE-PRA and pHAGE-PRB plasmids

pHAGE-PRA and pHAGE-PRB (Fig9 and 10) were a gift from Dean P. Edwards Lab Baylor College of Medicine, USA. pHAGE-PRA and pHAGE-PRB had 13 kb plasmids which compose of hPR (human progesterone receptor isoform A or B), rtTA (reverse tetracycline-controlled transactivator), and the ampicillin resistant gene. The function of rtTA induced the PR expression by tetracycline dependent (Tet-on system). The tetracycline analog, doxycycline (Dox) was binded with rtTA and activated the TRE (tetracycline-responsive promoter element) which induced PRA or PRB expression. (Fig8)
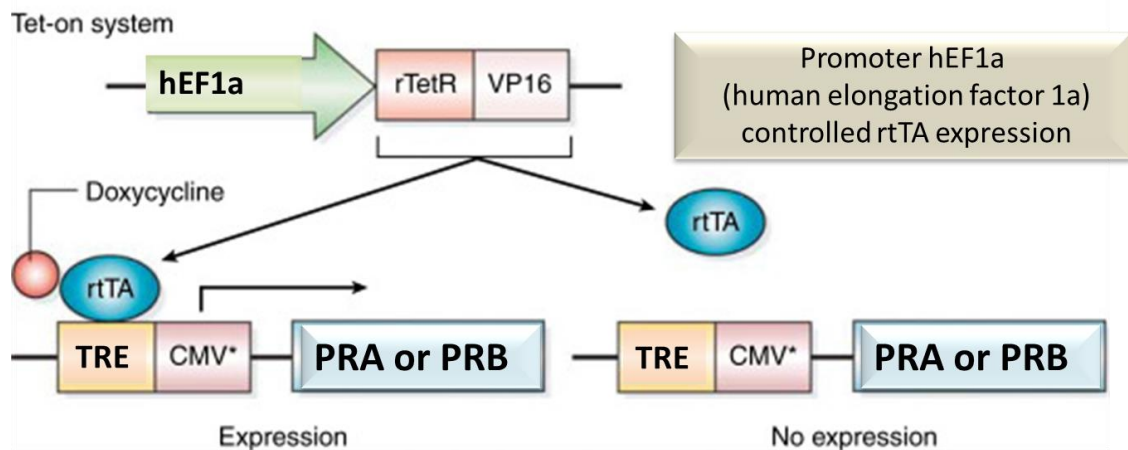
Figure 8 Tetracycline inducible PRA and PRB expression (94)
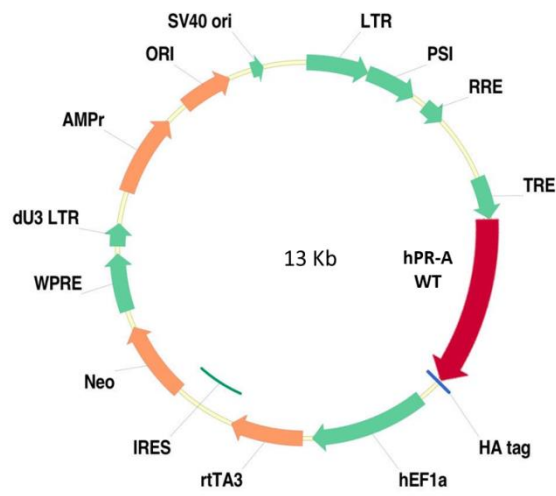
Figure 9 Plasmid map of pHAGE-PRA
(Dean P. Edwards Lab Baylor College of Medicine)
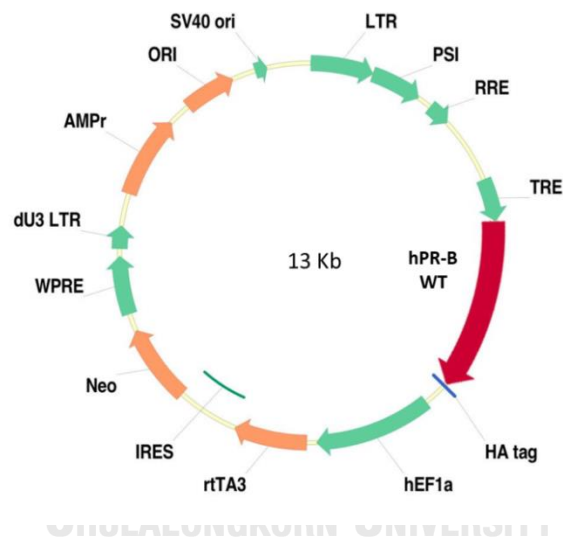
Figure 10 Plasmid map of pHAGE-PRB
(Dean P. Edwards Lab Baylor College of Medicine)

2. Plasmids for lentiviral particles production (psPAX2, pMD2.G)

psPAX2 had 10.7 Kb which using for viral packaging. paPAX2 plasmid consist of Gag, Pro, and Pol genes that encode the structural proteins and enzymes which showed in Fig11. While pMD2G (envelope plasmid) had 5.8 Kb which contains vesicular stomatitis virus (VSV-G) gene for envelope development showed in Fig12.
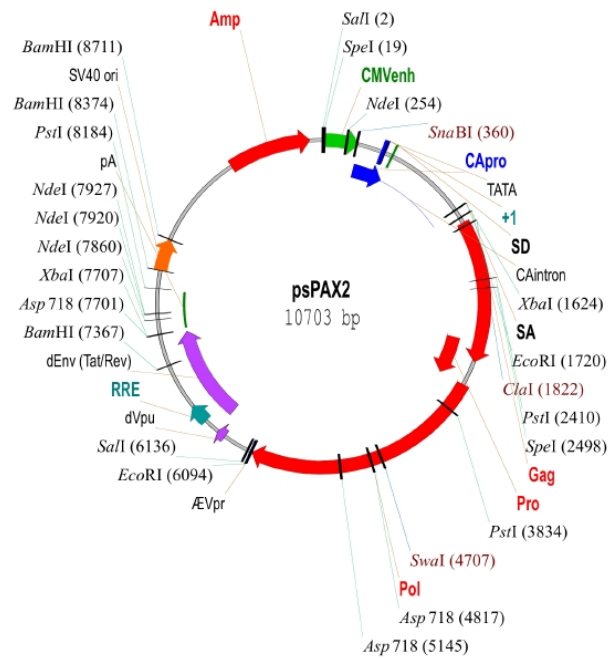
Figure 11 Plasmid map of psPAX2 plasmid (http://www.addgene.org/12260/)
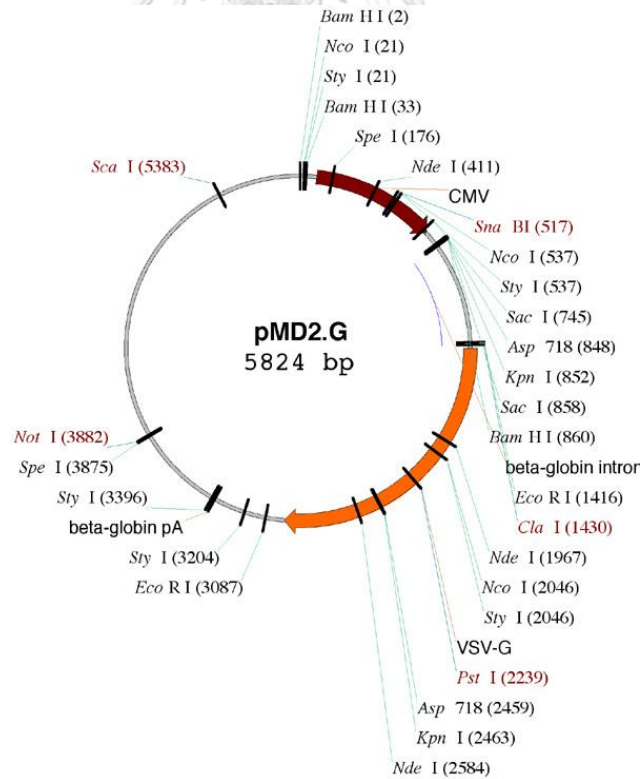
Figure 12 Plasmid map of pMD2.G plasmid (http://www.addgene.org/12259/)

Cells for cell culture

1. H727 lung neuroendocrine tumor cell line was a gift from Department of Pathology, Tohoku University Graduate School of Medicine, Japan. H727 cell line is a bronchial carcinoid cell line which expresses mRNA of p53, has EGF receptors, no PR expression and was used to construct tet-inducible PR expression.

2. T47D cell line is a human ductal breast epithelial tumor cells was a gift form Diana C.Marquez-Garban Department of Hematology/Oncology, David Geffen School of Medicine, USA. T47D was used as a positive control of PR. T47D is a constitutive PR expression which has high PR protein expression both A and B isoforms.

3. HEK293T cells line is a human embryonic kidney cell line 293T. HEK293T was used for transfected plasmid and produced lentiviral particles.

Tissue Collection

1. Lung neuroendocrine Tumor patients

Lung neuroendocrine tumor tissues were resected 198 cases between 1996 and 2015 from 8 hospitals in Japan; Aomori Prefectural Central Hospital 26 cases, Miyagi cancer center 28 cases, Ishinomaki Red Cross Hospital 19 cases, Iwate Prefectural Isawa Hospital 15 cases, Iwate Prefectural Central Hospital 36 cases, Tohoku University 27 cases, Tohoku Medical and Pharmaceutical University 34 cases and Sendai Medical Center 13 cases. Each cases of lung neuroendocrine tumor were obtained information of age, sex, tumor size, status of dead and alive, recurrence, treatment and stage from medical record. Lung neuroendocrine tumor types were diagnosed based on WHO classification 2015 criteria (5) with the detection of at least one of the neuroendocrine markers including chromogranin A, synaptophysin, and CD56 were used to confirm a diagnosis of lung NET (31). The results were achieved by pathologists (A.K and H.S.). We defined overall survival from initial pathological diagnosis to the time of death or last censor. Ethics Committee of Tohoku University Graduate School of Medicine approved the protocol of this study.

2. Normal fetal and adult lung

We also obtained normal fetal 9 cases (13-18 weeks) and adult lung 9 cases in bronchi area which achieved from Tohoku University. Normal lung tissues were serially resected to compare staining between SYN and double immunohistochemistry of SYN with PR or PRB. Ethics Committee of Tohoku University Graduate School of Medicine approved the protocol of this study.

Reagents

Reagents for determine RNA and protein expression of PR gene in H727 cell line by real-time RT-PCR

Genezol reagent	Geneaid, Taiwan
Accupower RT Premix	Bioneer, Korea
Chloroform	Sigma Aldrich, U.S.A

Reagents for DNA plasmid amplification

Competent cells E.coli strain DH5α	Real Biotechnolog,U.S.A
Tryptone	Thermoscientific, U.S.A
Yeast extract	Thermoscientific, U.S.A
NaCl	Merck Millipore, Germany
Agar	Oxiod, United Kingdom
Isopropanol	Sigma Aldrich, U.S.A
Ethanol	Merck Millipore, Germany
DNA ladder 1000 bp	Gene direx, U.S.A
Plasmid Maxi kits	Qiagen, Germany
Restric enzyme:	Thermoscientific, U.S.A
EcoRI, Ndel ,and SalI	

Reagents for Lentiviral production and cells model construction

Opti-MEM I medium	Gibco/Life Technologies, U.S.A
X-tremeGENE HP DNA transfection	Roche, Mennheim, German

Polybrene trabsfection reagent   Merck Millipore, Germany

G418   Gibco/Life Technologies, U.S.A

HEPES buffer   Hyclone Laboratories, U.S.A

Trypan blue   Gibco/Life Technologies, U.S.A

Doxycycline   Merck Millipore, Germany

Reagents for validated PRA and PRB protein expression in H727-PRA and H727-PRB cells model by western blot analysis

RIPA lysis buffer   Merck Millipore, U.S.A

Proteinase inhibitor cocktail   Roche, Mannhein, Germany

Standard protein Bovine serum   Merck Millipore, U.S.A
albumin (BSA)

Bradford dye reagent   Bio-Rad, U.S.A

Precision Plus Protein Standards   Bio-Rad, U.S.A
ladders

Blotting-Grade Blocker   Bio-Rad, U.S.A

1294 mouse monoclorial antibody   Dean Edwards, Baylor College
of Medicine

Anti-mouse IgG,l HRP linked Ab 7076s   Cell Signal Technology, U.S.A

Pierce® ECL WesternBlotting Substrate   Thermoscientific, U.S.A

Reagents for validated PRA and PRB protein localization in H727-PRA and H727-PRB cells model and characterized PRB specific antibody by Immunofluorescence

R5020   PerkinElmer, U.S.A

Paraformaldehyde   Sigma Aldrich, U.S.A

TritonX-100   Sigma Aldrich, U.S.A

Prolong® Gold antifade reagent   Invitrogen, Carlsbad, U.S.A

250H11 mouse monoclonal antibody   Characterized by Dean Edwards,
Baylor College of Medicine

Reagents for validated PRA and PRB protein transcriptional activity in H727-PRA and H727-PRB cells model by Luciferase Assay

TurbofectTM Kit	Thermoscientific, U.S.A
Dual-Glo® Luciferase Assay System	Promega, U.S.A
Lysis reagent 5x	Promega, U.S.A

Reagents for determine cells viability by MTT assay

MTT	Applichem, Germany
SDS	Bio-Rad, U.S.A
RU486	Sigma Aldrich, U.S.A

Reagents for cell culture

DMEM	Hyclone Laboratorie, U.S.A
RPMI	Hyclone Laboratorie, U.S.A
Fetal bovine serum (FBS)	Merck Millipore, Germany
Charcoal Stripped Fetal Bovine Serum	Thermoscientific, U.S.A
Penicillin Streptomycin	Hyclone Laboratorie, U.S.A
EDTA-Trypsin 0.25% (1x)	Hyclone Laboratorie, U.S.A
Phosphate Buffered Saline (PBS)	Hyclone Laboratorie, U.S.A
Trypan blue	Gibco, U.S.A

Reagents for Immunohistochemisty and double immunohistochemistry

Xylene	Tohoku University, Japan
Alcohol	Tohoku University, Japan
Methanol	Tohoku University, Japan
Citrate buffer	Tohoku University, Japan
Histofine kits	Nichirei Bioscience, Japan
DAB solution	Tohoku University, Japan
Vector blue	Vector Laboratories, U.S.A

Materials and Equipment

-20°C Freezer	Sanryo Electric, Japan
-80°C ULT Deep Freezer	IlShin Lab, South Korea
4°C Refrigerator	Sharp, Japan
6 well cell culture plate flat bottom with lid	Nunc, Denmark
12 well cell culture plate flat bottom with lid	Nunc, Denmark
96 well cell culture plate flat bottom with lid	Nunc, Denmark
Auto pipette	Gilson, France
Block heater	Wealtec Corp., U.S.A
Biotek Synergy Mx microplate reader	Biotek, U.S.A
Cell Culture Flask (25 cm2)	Nunc, Denmark
Centrifuge tube 15, 50 ml	Corning Inc, U.S.A
CO2 incubator	Sheldon Manufacturing, U.S.A
Cryovial tube 2.0 ml	Nunc, Denmark
Confocal microscope LSM700	Carl Ziess, Germany
Disposable Serological pipette (2, 5, 10 ml)	Corning Inc,. U.S.A
Electrophoresis power supply	Bio-Rad Laboratories, U.S.A
Gel documentation (gel doc) systems	Syngene, England
Gel Electrophoresis Apparatus	Bio-Rad Laboratories, U.S.A
Glassware	Pyrax, U.S.A
Hermocytometer	Hausser scientific, U.S.A
Incubator	Memmert, Germany
Inverted microscope	Olympus Optical, Japan
Larminar Flow Cabinet	E.S.I. Flufrance, France
Light microscope	Olympus Optical, Japan
Liquid Nitrogen Tank	Taylor-Wharton,
Lumiometer Plate	PerkinElmer, Finland

Microcentrifuge	Denver Instrument, U.S.A
Microcentrifuge tube (1.5 ml)	Bio-rad, U.S.A
Micro Refrigerated Centrifuge	Vision Scientific, South Korea
Multichannel pipette	Brand, Germany
Pipette tips (10, 200, 1000 µl)	Biobasic, Canada, Biorad, U.S.A
Thermal Cycler	MU Research Inc., U.S.A
Vacuum Concentrator	Thermo Electron Corporation,
(DNA SpeedVacs)	U.S.A
Vortex Mixer	Finepcr, South Korea
Water Bath	Memmert, Germany

Water used for research was Milli-Q™ which passed through 18 mΩ voltage of ultraviolet light and was filtered through 0.22 µm pores.
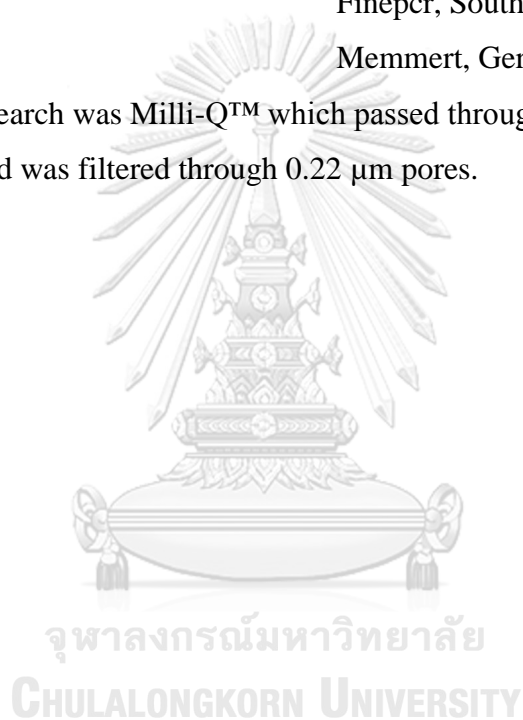
Methods

1.   Determination of PR RNA and protein expression in H727 cell line by real-time RT-PCR and western-blot analysis.

1.1 Quantitative reverse transcription PCR RT-qPCR

Principle: Quantitative reverse transcription PCR (RT-qPCR) started with RNA. Total messenger RNA (mRNA) was converted to complementary DNA (cDNA) by reverse transcriptase. Then cDNA was used as a template for real time PCR (qPCR) reaction. In PCR process consists of three stages; denaturation, annealing, and elongation. Then SYBR GreenI was bound to any double-stranded DNA. The fluorescence was measured real time in each cycle (95).

H727 and T47D (positive control) cells line were cultured in 6-well plate. H727 cell line was cultured 3.0×10^5 in RPMI with 10% FBS and 1% Penicillin Streptomycin. T47D cell line was cultured 2.5×10^5 in DMEM with 5% FBS and 1% Penicillin Streptomycin. Cells were cultured 72 hours to get 80-90% cell confluence. Then cells were lysed, first, medium was removed then rinsed cells with 1 ml cold PBS 2 times and added Genezol reagent 1 ml, then incubated 5 minutes at room temperature. Cells were mixed by pipetting up and down and transferred cells lysates to 1.5 ml tube. Cells were stored at -80°C. Next day, H727 and T47D cell lysates were used to extract RNA by using Chloroform 200 µl. Lysates and chloroform were invert mixed and centrifuged the mixture at 15,000 g for 15 minutes at 4°C. Aqueous phase was collected to another 1.5 ml tube. Isopropanol was used to precipitate RNA by added isopropanol and incubated at room temperature for 10 minutes, centrifuged at 15,000 g, 15 minutes at 4°C. RNA pellets were washed 3 times with 70% ethanol. Then RNA pellets were dried at room temperature for 2-3 hours and dissolved pellets by Rnases-free water. Total RNA was measured by Nano drop at OD 260/280. In the next day, cDNA were generated from one microgram of total RNA using Accupower RT Premix. 1 µg of RNA was mixed with 0.5µg of Oligo dT$_{18}$ and incubated at 70°C for 5 minutes. Tubes were moved on ice then filled up to the 20µl reaction volume with Rnase-free water. Then performed cDNA synthesis reaction by Bioneer exicycler^{TM}96 at $42 \circ$C, 60 min and $94 \circ$C, 5 min. Samples were kept on $-20 \circ$C. The

next day, cDNA was analyzed by real-time PCR with PR gene primer (Forward primer: 5'TGG AAG AAA TGA CTG CAT CG3', Reverse primer: 5'TAG GGC TTG GCT TTC ATT TG3') with PCR product 195 bp (11) and GAPDH gene primer (Forward primer: 5'ACA TCG CTC AGA CAC CAT G3', Reverse primer: 5'TGT AGT TGA GGT CAA TGA AGG G3') (96). PR and GAPDH were performed by using Green Star PCR Master Mix and analyzed by Bioneer exicycler^{TM}96 with reaction of pre-denaturation 95°C for 10 minutes, Denaturation95^{o}C for 15 seconds, PR Annelaing 53°C for 30 seconds and GAPDH annealing 57°C for 30 seconds with 34 cycles. The PCR products of PR gene were also analyzed by gel electrophoresis 100 volts 35 minutes in 2% agarose gel (agarose 2 g with TAE buffer 100 ml). Bands were visualized by Gel documentation (Gel doc) systems. Samples without RNA templates were used as negative control to confirm genomic DNA contamination.

1.2 Western-blot Analysis

Principle: Western blot was used for protein detection. Proteins were load and separated into polyacrylamide gel according to their molecular weight. Then transferred protein from gel to nitrocellulose membrane and detected protein by specific primary antibody, secondary antibody with enzyme labeled and substrate. Proteins were visualized by chemiluminescence (97).

H727 cell line was seeded in 6-well cultured plate 3.0×10^5 cells per well and incubated for 48 hours. T47D cell was seeded 7.5×10^5 cells per well in 6-well plate and incubated 24 hours for 80-90% cells confluence. Then cells were lysed by lysis buffer of 1x RIPA lysis buffer (10x RIPA lysis buffer consists of 0.5M Tris-HCL pH7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10%NP-40, 10mM EDTA) and proteinase inhibitor cocktail or PIs (consists of serine protease, esterases, cysteine proteases, metalloproteases, trypsin-like proteases). Ratio of PIs : RIPA buffer was 1:7. RIPA lysis buffer was added 100µl per well in 6-well plate. Lysis buffer and cells were incubated on ice for 5 minutes than used scraper scraped cells in the well. Cell lysates were transferred to 1.5 ml tube and centrifuged at 8000 g, 10 minutes at 4°C. Supernatant was collected and stored at -80°C.

Total protein was measured by Bradford assay. Bovine serum albumin (BSA) concentration of 0.5, 0.25, 0.125, 0.06, 0.03 and 0 mg/ml was used as standard,

Bradford dye reagent (Bradford dye, phosphoric acid, methanol) diluted with milliQ water of ratio 1:5, Blank sample (lysis buffer diluted with milliQ water 1:15) and protein samples (diluted with milliQ water 1:15). BSA standard and protein samples were added 10 µl in 96-well plate then added dye reagent 200 µl and measured at OD of 595 nm. Results were plot in graph and calculated protein concentration for our samples.

After measured proteins, proteins were run on a 10% SDS-PAGE gel. Glass plates were put on both side of SDS-PAGE gel set up cassette. 10% Separation gel was dropped between 2 glass plate until volume was two third of height of the glass plate. Water was filled for removed bubbles. Wait until separation gel was polymerized which around 13 minutes then removed water from glass plates. 6% Stacking gel was dropped next until it is full and put comb in stacking gel immediately. After the gel was polymerized the comb was removed. The gel with the glass plates were soaked inside 1x Running buffer (10x running buffer 100 ml, 20%SDS 5 ml, milliQ water 900 ml). Proteins were calculated and used in total with DW 22.5 µl mixed with leamli 7.5 µl then heated 95°C for 10 minutes and loading samples including H727 protein and T47D (positive control) protein 30 µl and precision Plus Protein Standards ladder 4 µl. Proteins were separated in stacking gel 70 volt 30 minutes and separating gel 120 volt 90 minutes.

After electrophoresis, the separated proteins are transferred, or "blotted", onto a polyvinylidene difluoride (PVDF) membrane. PVDF membrane was activated by soak in methanol 5 minutes then transferred to soak in transfer buffer. The gel is then placed in the "transfer sandwich" (filter paper-gel-membrane-filter paper), cushioned by pads and pressed together by a support grid. Then run 150 mAmp 90 minutes by transfer western blot. The membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature, and washed with TBST buffer 3 times (1 M Tris-base pH 7.4, 5 M NaCl, 0.05% Tween-20). Then blots were incubated with primary antibody recognizing PR (1294 PR, a specific mouse monoclonal antibody) (98)dilution 1:2500 which detected PR isoforms A and B, or GAPDH antibody (Santa Cruz Biotechnology, CA, USA) dilution 1:10000 v/v overnight at 4 °C. After incubation with primary antibodies, blots were washed 3 times with TBST buffer, and incubated with secondary antibodies anti-mouse IgG HRP-linked antibody (7076s: Cell

Signaling Technology, Danvers, USA) or anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Danvers, USA) at room temperature for 1 hour then washed TBST 3 times.

Protein bands were detected on X-Ray film by chemiluminescence reaction using Pierce® ECL Western Blotting Substrate. Substrate working solutions was used as ratio 1:1. Substrate was added to PVDF membrane. Then moved membrane to the film cassette, PVDF membrane was placed at bottom, plastic and X-ray film on the top and incubated 1-5 minutes. X-ray film was exposed in Developer, water, Fixer and water respectively. Films were dried at room temperature. T47D was used as positive controls for PR expression (99, 100) and GAPDH was used as loading control.

2.   Lentiviral Particles production

All of the plasmids will be transformed into *E.coli* strain DH5α by heat shock transforrmation. First, 100-200 ng/ml of plasmid will be added to the competent cells and gently mixed for a few times. Second, plate the competent cell/plasmid mixture on ice 20 minutes and then heat shock at 42 ℃ for 2 minutes. Third, added 350 μl of SOC medium and grow at 37℃ in shaking incubator for 2 hours and then plate some or all of the transformation into Luria Bertini broth (LB) agar with ampicillin and incubated at 37℃ overnight. Select the isolated colony and culture in LB with 100 μg/mL ampicillin, follow by the incubation of LB broth at 37℃, 225 rpm for 14-16 hrs. The plasmid will be extracted via alkaline lysis by using plasmid Maxi kits, containing buffer P1(50mM Tris-Cl, pH 8.0, 10mM EDTA, 100 μg/mL RNase A) for cell resuspension, buffer P2 (200mM NaOH, 1% SDS) for breaking cell membrane, and buffer P3 (3.0 M potassium acetate, pH 5.5) for neutralizing pH. Finally, the extracted plasmid will be purified through the Qiagen tip. After plasmid extraction and purification, to validated plasmid DNA, all of plasmids were cut with restriction enzymes. pHAGE-PRA and pHAGE-PRB were cut by *Nde*I and *Sal*I and psPAX2 and pMD2G were cut by *Eco*RI, then incubated at 37℃ for 2 hours. Then separated DNA by gel electrophoresis 100 volt 40 minutes and detected bands by Gel documentation (gel doc) systems. (Plasmid amplification, extraction and purification

procedure was done by Miss Prangwan Pateetin, Miss Panthita Kaewjanthong and Miss Nattamolphan Wittayavimol)

HEK293T cells were seed 2.0×10^5 cells per well in 6-well tissue culture plate in DMEM with 10% FBS and 1% Penicillin Streptomycin was used to generate lentiviral particles. After 24 hours of 50-60% HEK293T cells confluence, cells were treated with with Opti-MEM I medium mixed with packaging plasmid (psPAX2), envelope plasmid (pMD2G), a DNA construct (pHAGE-PRA or pHAGE-PRB), and X-tremeGENE HP DNA. The ratio of lentiviral DNA construct: psPAX2: pMD2G was 1:2:2, and the ratio of the DNA construct: XtremeGENE HP DNA transfection reagent was 1:3.5 as Table1. All mixtures from Table 1 were added to HEK293T cells 200 µl in each well.

DNA construct	DNA (ug)	pHAGE-PRA (437 ng/ml)	pHAGE-PRB (634 ng/ml)
Lenti-Retro construct	0.57	1.34 µl	0.899 µl
psPAX2 (1163 ng/ml)	1.00	0.86 µl	0.86 µl
pMD2G (1157 ng/ml)	0.55	0.475 µl	0.475 µl
Opti-MEM medium		200 µl	200 µl
X-tremeGENE		3.5 µl	3.5 µl

Table 1 Components for lentiviral particles production

Media containing viral particles were collected after incubated 48 hours and stored at 4°C then added 2 ml of medium to each well. After cell transfection 72 hours, medium containing viral particles were collected again. Medium was filtered with 0.45 µM sterile filter PVDF membrane sand stored at -80 °C (101).

3.   Construction of tet-inducible PRA and PRB protein expression in H727 cells model.

H727 were seeded at 5.0×10^5 cells in tissue culture dish 100X20 mm and to achieve 50-60% confluency after incubated for 24 hours. Then cells were treated with

2.5 ml of DMEM medium containing pHAGE-PRA or pHAGE-PRB viral particles, mixed with 2.5 ml serum-free RPMI medium and 8 μg/ml of polybrene transfection reagent 4 μl, respectively. The cells with medium containing virus were incubated at 37°C for 4 hours. After incubation period, 5 ml of RPMI with 10% FBS was added and cell were incubated with viral particles for overnight. The next day, medium was changed to 10 ml RPMI supplemented with 10% FBS then incubated addition 48 hours. H727 viral infected cells were selected in RPMI medium with 10%FBS containing G418 500 μg/ml for 14 days (condition was tried by Miss Sornsawan Kawprasertsri). Dox was added to induce PRA and PRB protein expression in H727-PRA and H727-PRB cells model (102). Then cell models were validated PRA or PRB protein expression, localization and transcriptional activity.

4. Validation of tet-inducible PRA and PRB protein expression in H727-PRA and H727-PRB cells model by western blot analysis.

5.1 Determination of doxycycline optimal concentration for maximum induction of PRA and PRB protein expression.

After H727 cells line was transfected with lentiviral particles and selected with G418, H727-PRA and H727-PRB cells model were treated with doxycycline in different concentration (0, 50, 200, 500, 700 and 1000 ng/ml) for 24 hours to determine optimal concentration for maximum PRA and PRB protein expression. T47D was used as positive control and GAPDH was used as loading control. After 24 hours, cells were lysed by lysis buffer and analysed by western blot analysis as previously describe (1.2)

5.2 Determination of doxycycline optimal time for maximum induction of PRA and PRB protein expression.

H727-PRA and H727-PRB cells model were treated with doxycycline optimal concentration for maximum PRA and PRB protein expression from 4.1 in different time 0, 24, 48, 72 and 96 hours to determine optimal time for maximum PRA and PRB protein expression. T47D was used as positive control and GAPDH was used as

loading control. After 24 hours, cells were lysed by lysis buffer and analysed by western blot analysis as previously describe (1.2)

5. Validation of PRA and PRB protein localization in H727-PRA and H727-PRB cells model and characterization of PRB (250H11) specific antibody by immunofluorescence.

Principle: Immunofluorescence is an assay which detects antigens in cellular contexts using specific primary antibody and fluorescent labeled secondary antibody then visualized by fluorescence microscope (103).

H727-PRA, H727-PRB were seeded 3.5×10^5 per well in 6 well tissue culture plate with 3 coverslip in each well at 37 °C overnight to determine PRA and PRB proteins localization. Then treated the cells with Dox 700 ng/ml for 24 hr to induce maximum PR expression and then treated with 10 nM progestin agonist R5020 for 1 hour. After that, cells were washed with cold PBS at PH 7.4 and transfer cover slip to 12-well plate. Then fixed cells in 4%paraformaldehyde for 20 minutes, washed with PBS 3 times, add triton X-100 10 minutes, washed with PBS 3 times and blocked with 1% FBS-PBS for 1 hour. Then incubated cells with primary for PR(1294 PR, a specific mouse monoclonal antibody) dilution 1:3000 and PRB (250H11 PRB-specific mouse monoclonal antibody obtained from Dead Edward, Baylor College of Medicine) dilution 1:1000 in 1%BSA-PBS (v/v) at 4˚C overnight. Before add secondary antibody, cells were washed with PBS and treated with secondary antibody, rabbit anti-mouse IgG-Alexa568 at 1:5000 in 1%BSA-PBS (v/v) for 1 hour in room temperature. Washed coverslip with PBS 3 times then incubated with DAPI (4',6-diamino-2-phenylindole) at 1:5000 in PBS (v/v) for 10 minutes. PBS was used to wash the cells and Prolong® Gold antifade reagent was used to mount cells on microscope slides. LSM700 laser confocal scanning microscope was used to analyze localization of PRA and PRB proteins in H727-PRA and H727-PRB cells model. Fluorophores of DAPI were excited with a 405-nm laser and 561-nm for Alexa568. The images were analyzed by ImageJ v 1.50 software (National Institute of Health, USA) Intensity of fluorescence for each cell was obtained by selected area of interest around the whole cell or nucleus PR and PRB staining.

6.   Validation of PRA and PRB protein transcriptional activity in H727-PRA and H727-PRB cells model by luciferase assay.

Principle: Luciferase reporter assay is used for assess transcriptional activities in the cells. Cells were transfected with a genetic construct containing luciferase gene under the control of progesterone response element. Transcriptional activity was detected by measure luciferase reaction, after adding luciferin as a substrate. Photon emission was detected by luminometer (104).

Transcriptional activities of PRA or PRB proteins in H727, H727-PRA and H727-PRB cells were examined. Cells were seeded at 1.0×10^5 cells per well in 24-well plate in RPMI supplemented with 5% DCC-FBS at 1% PenStrep in 24 well-dish incubated overnight. Then cells were transfected with 50 ng pRL-CMV (Renilla) and 450 ng PRE2-TK Luciferase reporter construct (105). (Plasmid amplification, extraction and purification procedure was done by Miss Prangwan Pateetin, Miss Panthita Kaewjanthong). Transient transfections were performed by using the TurboFect™ Transfection. After 24 hours, cells were treated with Dox 700 ng/ml for 24 hours and treated with 10 nm R5020 or ethanol (vehicle control) for an additional 24 hours. Then cells were lysed by lysis buffer on ice, cell lysates were collected and centrifuged at 8,000g, 4℃ for 10 minutes. Luciferase activities were analyzed supernatant of cell lysates 30 μl in 96-well plate using the Dual-Glo® Luciferase Assay System. Dual luciferase reagent was added 30 μl and incubated for 10 minutes then measured firefly luciferase activity. After that, used the same 96-well plate, added Stop Glo buffer 30 μl and incubated for 10 minutes then measured renilla luciferase activity. Ratio of firefly luciferase and renilla luciferase were calculated. Relative luciferase activity was calculated as firefly luciferase $\times$ 100 divided by renilla luciferase. Value are represented as means±SEM from three triplicates (n=3).

7.   Determination of cell viable in H727-PRA and H727-PRB cells model after express PRA and PRB, respectively, by MTT assay.

Principle: MTT assay is a colorimetric assay for assess metabolic activity in the cells. Cellular oxoredutase enzymes may reflect the number of viable cells. Enzymes are capable of reducing tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide to its insoluble formazan (purple color). Then insoluble formazan were dissolved by SDS. Optical Density at 570 nm was used to measured formazan product (106).

7.1 Determination of cell viability in H727-PRA and H727-PRB cells model in the absence of ligand.

7.1.1 Determination of optimal concentration for maximum PRA and PRB effects.

H727, H727-PRA, and H727-PRB were seeded in 96-well plate at 1.5×10^4 cells per well in RPMI supplemented with 5% DCC-FBS 1% PenStrep in a 96 well tissue culture plate and incubated for 24 hours to achieved 50-60% confluence. Then cell were treated with Dox in an increasing concentration (0, 50, 200, 500, 700 and 1000 ng/ml) for 48 hours to determine optimal concentration for maximum PRA and PRB effects. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 5 mg/ml was added 10 µl and incubated 37°C 3 hours. The insoluble formazan was dissolved by 10% sodium dodecyl sulfate (SDS) 100 µl in each well overnight. The absorbance was measured at 570 nm by microplate. All experiments were carried out in triplicates which are representative data from three independent experiments as means ± SEM. Results were presented as % cell viability compared with the control value of each experiment.

7.1.2 Determination of optimal time for maximum PRA and PRB effects.

H727-PRA, and H727-PRB were seeded in 96-well plate at 1.5×10^4 cells per well in RPMI supplemented with 5% DCC-FBS 1% PenStrep in a 96 well tissue culture plate and incubated for 24 hours to achieved 50-60% confluence. Then cell were treated with Dox 700 ng/ml in following time; 0, 24, 48 and 72 hour. MTT 5 mg/ml was added 10 µl and incubated 37°C 3 hours. The insoluble formazan was dissolved by 10% sodium dodecyl sulfate (SDS) 100 µl in each well overnight. The absorbance was measured at 570 nm by microplate. All experiments were carried out in triplicates which are representative data from three independent experiments as means ± SEM. Results were presented as % cell viability compared with the control value of each experiment.

7.2 Determination of cell viability in H727-PRA and H727-PRB cells model in the presence of ligand.

To determine the effects of progestin and anti-progestin, H727-PRA, and H727-PRB were seeded in 96-well plate at 1.5×10^4 cells per well in RPMI supplemented with 5% DCC-FBS 1% PenStrep in a 96 well tissue culture plate and incubated for 24 hours to achieved 50-60% confluence. Cells were pretreated with Dox 700 ng/ml for 24 hours, then treated with 10 nM of R5020, 100nM of RU486 and combination of R5020 and RU486 (107) then incubated and measured at 0, 24, 48, and 72 hours. After treatment, MTT solution (5 mg/ml) 10 µl was added to each well and incubated for 3 hours. The insoluble formazan was dissolved by 10% sodium dodecyl sulfate (SDS) 100 µl in each well overnight. The absorbance were measured at 570 nm by microplate spectrophotometer and All experiments were carried out in triplicates which are representative data from three independent experiments as means ± SEM. Results were presented as % cell viability compared with the control value of each experiment.

8.   Examination of PR and PRB expression in lung neuroendocrine tumors tissues by immunohistochemistry.

Principle: Immunohistochemistry is a method for detected antigens of tissue section by specific antibody. The antigen-antibody binding could be visualized by Horseradish Peroxidase (HRP) enzyme (108).

The specimens were formalin-fixed and paraffin-embedded. For PR/PRB immunohistochemistry were used streptavidin-biotin amplification method by Histofine Kit. The specimens in the paraffin blocks were cut into 3-µm-thick sections and mount on APES-coated slides, de-paraffinize and re-hydrate in xylene and alcohol respectively. Then did antigen retrieval by treated with citrate buffer (2 m M citric acid and 9 m M trisodium citrate dihydrate, pH 6.0) and autoclaved at 121°C for 5 min. After antigen retrieval, samples were incubated with rabbit-blocking solution at room temperature for 30 min. Slides were incubated with primary antibody recognizing PR and PRB (1294 PR-specific mouse monoclonal antibody,1:1000) (98), recognizing PRB (250H11 PRB-specific mouse monoclonal antibody, 1:2000),

Chromogranin A (DAKO, Tokyo, Japan), Synaptophysin (DAKO, Tokyo, Japan) CD56 (Nichirei Bioscience INC, Tokyo, Japan) and Ki67 (DAKO, Tokyo, Japan) at 4ºC overnight. (Morphological evaluation and immunohistochemistry of Chromogranin A, Synaptaphysin and Ki-67 were done by Dr. Atsuko Kasajima). Peroxidase-conjugated streptavidin was used to incubate for 30 min and secondary antibody was used to incubated next for 1 hour at room temperature. Antigen-antibody complex were visualized with 3,3′-diaminobenzidine tetrahydro-chloride solution (1 m M DAB, 50 m M Tris-HCl buffer, pH 7.6, and 0.006% H_2O_2) (DAB) and then counterstained with hematoxylin. Negative controls were obtained with rabbit immunoglobulin in the same concentration with the samples of lung neuroendocrine tumors.

9.   Examination of PR and PRB localization in pulmonary neuroendocrine cells (PNECs) of normal fetal and adult lung tissues by double immunohistochemistry.

Principle: Double immunohistochemistry is a method for detected antigens of tissue section by 2 specific antibodies. The first antigen-antibody binding could be visualized by Horseradish Peroxidase (HRP) enzyme and the second antigen-antibody complex was visualized by alkaline phosphatase (109).

Fetal and adult normal lungs were fixed in 10% formalin and embedded in paraffin wax. The specimens in the paraffin blocks were cut and mount on APES-coated slides, de-paraffinize and re-hydrate in xylene and alcohol respectively. Antigen retrieval were treated with citrate buffer (2 m M citric acid and 9 m M trisodium citrate dihydrate, pH 6.0) and autoclaved at 121°C for 5 min. Then incubated samples with rabbit-blocking solution at room temperature for 30 min then incubated with primary antibody recognizing SYN (Dako, Glostrup, Denmark; 1:5000) at 4ºC overnight. Slides were incubated with peroxidase-conjugated streptavidin for 30 min at room temperature and secondary antibody for 1 hour. For the first antibody, antigen-antibody complex were visualized with 3,3′-diaminobenzidine tetrahydro-chloride solution (DAB) (1 m M DAB, 50 m M Tris-HCl buffer, pH 7.6, and 0.006% H_2O_2). Then used the same slides to done antigen retrieval by treated with citrate buffer (2 m M citric acid and 9 m M trisodium citrate

dihydrate, pH 6.0) and microwave 20 min. The samples were incubated with rabbit-blocking solution in room temperature for 30 min. Then incubated with primary antibody recognizing PR/PRB (1294 PR, a specific mouse monoclonal antibody)(98)or recognizing PRB (250H11 PRB-specific mouse monoclonal antibody) at 4°C overnight. After, slides were incubated with biotin-labeled anti-mouse IgG and incubated with pre-form avidin-biotin-alkaline phosphatase complex. Vector Blue® (Vector Laboratories, Burlingame, CA) was used to visualize PR (1294) or PRB (250H11) antibody (blue) in contrast to SYN (brown).

10.   Scoring of Immunoreactivity

Identical microscopes (BX50; Olympus, Tokyo, Japan) were used for morphological, Double immunohistochemistry and PR (1294) and PRB (250H11) immunohistochemistry were analyzed by 2 observers (T.A and S.Y), CgA, SYN, and Ki-67 were analyzed by pathologist (A.K and H.S). We used high-power field ($\times$400) for evaluate Immunohistochemistry of PR and PRB also with double immunohistochemistry of SYN^+PR^+ and SYN^+PRB^+ and SYN^+. immunohistochemistry stained used H-score (110) for analyzed immunoreactivity of PR and PRB. The H-score was obtained by the formula: "3 x percentage of strongly staining nuclei + 2 x percentage of moderately staining nuclei + 1 x percentage of weakly staining nuclei which giving a range of 0 to 300" according to S Detre, et al, J Clin Pathol, 1995 (111) which H-score was >1% positive cells (112), were noted as PR/PRB positive. Double immunohistochemistry stained of SYN^+PR^+ and SYN^+PRB^+ were counted each field and used to compare with SYN^+ Immunohistochemistry. The SYN^+ was used to compare negative cells in epithelial cells of bronchi area. All of the normal lung staining were calculated in mean of ratio (113). Double immuhohistochemistry and H-score analysis were defined by >2000 tumor cells count.

11.   Statistical Analysis

Statistical analysis was done by The JMP Pro software version 13.0.1 (SAS Institute, Cary, N.C., USA) (114). Paired t-test or one-way ANOVA method were used to analyzed statistic as appropriated with bonferroni post-test correction. Value were carried out in triplicates which are representative data from three independent experiments as means ± SEM. Pearson's $\chi 2$-test was used to evaluated clinicopathological factors and immunoreactivity. Mann–Whitney U-test was used to analyze continuous variables. Survival analysis performed by Kaplan-Meier survival curves analyzed by log rank test and multivariate analysis was performed by Cox regression. Statistical significance was defined as P-value of less than 0.05 with two sided test.

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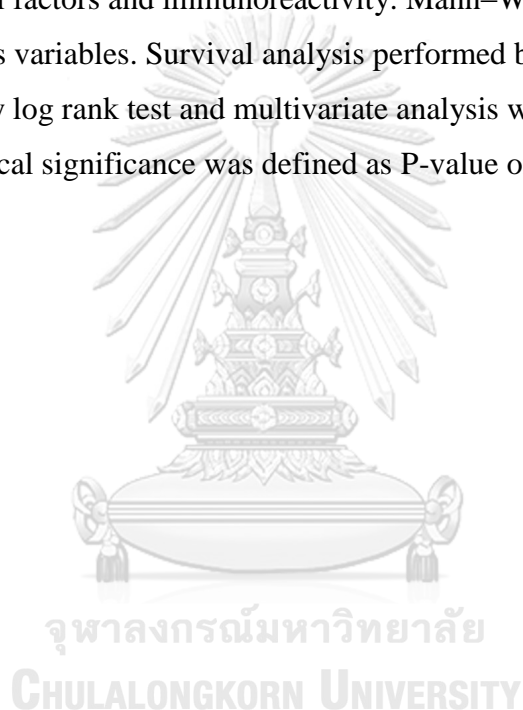
CHAPTER VI
RESULTS

1. Determination of PR RNA and protein expression in H727 cell line by real-time RT-PCR and western-blot analysis.

Several clinical studies showed that PR expression leads to better prognosis. *In vitro* studies, our previous study showed that PRB expression in NSCLC inhibits cell proliferation through epidermal growth factor signaling (11). However, prognosis associated with PR and PR isoforms expressions in lung NET are unknown. As first, a step toward understanding of the role of PR isoforms in lung NET, we constructed Tet-inducible PRA and PRB expression cell model in wild type H727, lung NET cell model. We first confirmed that H727 cells do not express both isoforms of PR by examining PRA and PRB mRNA by qRT-PCR (Fig13A) and gel electrophoresis (Fig13B) using PR specific primers. We also analyzed PR protein expression in wild type H727 cell line by western blotting (Fig13C) with PR (1294 mAb) specific antibody. T47D breast cancer cells were used as a positive control for PR expression at both mRNA and protein levels. We failed to detect PR (PRA and PRB) mRNA and protein expression in H727 lung NET cells but were able to detect PR mRNA and protein expression in T47D breast cancer cells. (Fig13A-13C) These data indicated that H727 do not express endogenous PR, allowing us to examine the role of PR isoforms by ectopically expressing PRA or PRB, without interference from endogenous PR.
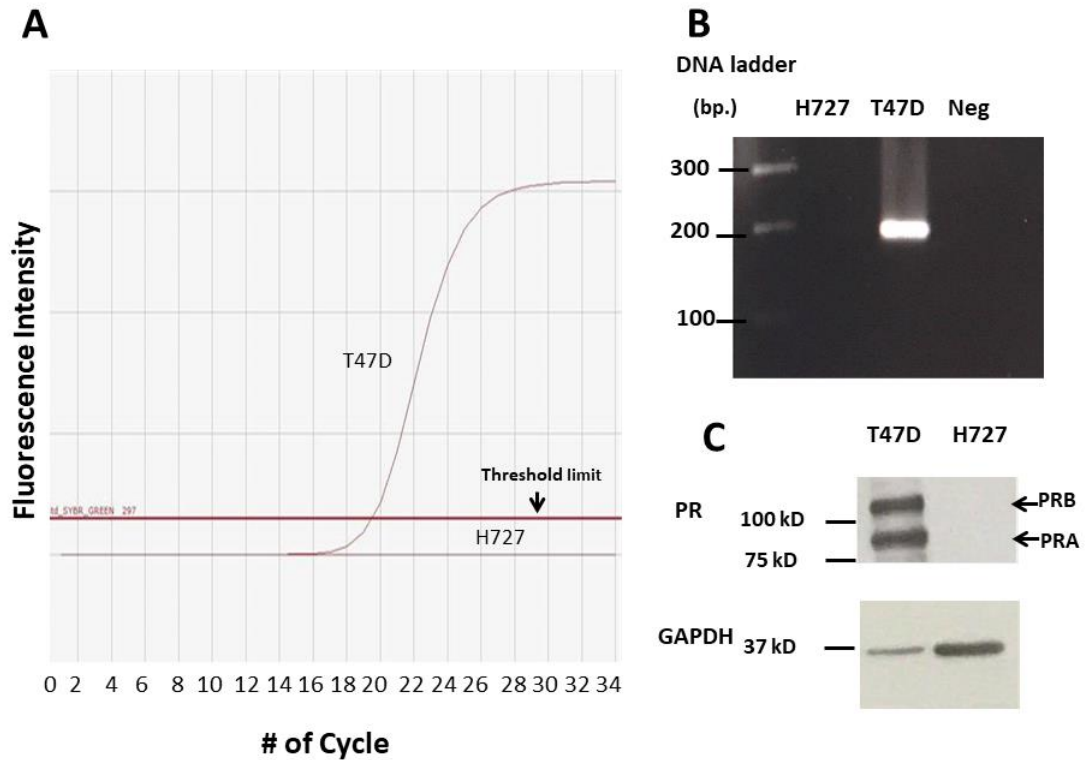
Figure 13 PR mRNA and protein expression in H727 and T47D cell lines (A) Total RNA were extracted from H727 and T47D cells, converted to cDNA and amplified with real time RT-PCR, results shown as amplification plot. (B) Products from RT-PCR were run on on 2% agarose gels gel electrophoresis: lane 1:100 bp DNA ladder, lane 2: H727 RNA, lane 3: T47D RNA (positive control) and lane 4: no RNA template negative control. (C)Western blotting were carried on 30 µg of cell lysates from H727 cells and 3 µg from T47D cells using PR-specific monoclonal antibody mouse monoclonal antibody (1294 mAb). Protein loading was normalized with GAPDH as an internal control.

2.   Validation of tet-inducible PRA and PRB protein expression in H727-PRA and H727-PRB cells model by western-blot analysis.

We constructed tetracycline-inducible PRA and PRB expression in H727 lung NET cells to examine how expression of PRA or PRB affected H727 biological properties. H727 expressing tet-inducible PRA or PRB (H727-PRA and H727-PRB) cell model were constructed transducing with lentiviral constructs expressing tet-inducible PRA or PRB as described in Materials and Methods.
We next used PR specific, 1294 mAb, to help validate expression of PR isoforms H727 cell model. H727-PRA and PRB cell model were treated with tetracycline analog (doxycycline,Dox) then lysed and analyze with PR (1294) specific antibody by western blotting. Doxycycline were treated with increasing concentration (0, 50, 200, 500, 700 and 1000 ng/ml) for 24 hours. PRA and PRB protein expression in H727 cell model were expressed the most when treated with doxycycline 700 ng/ml. (Fig14A and 14B) Then treated cells with doxycycline 700 ng/ml in different time course (0, 24, 48, 72 and 96 hrs) (Fig14C and 14D). PRA and PRB protein expression in H727 cell model were expressed the most when treated with doxycycline 700 ng/ml in 24 hours.
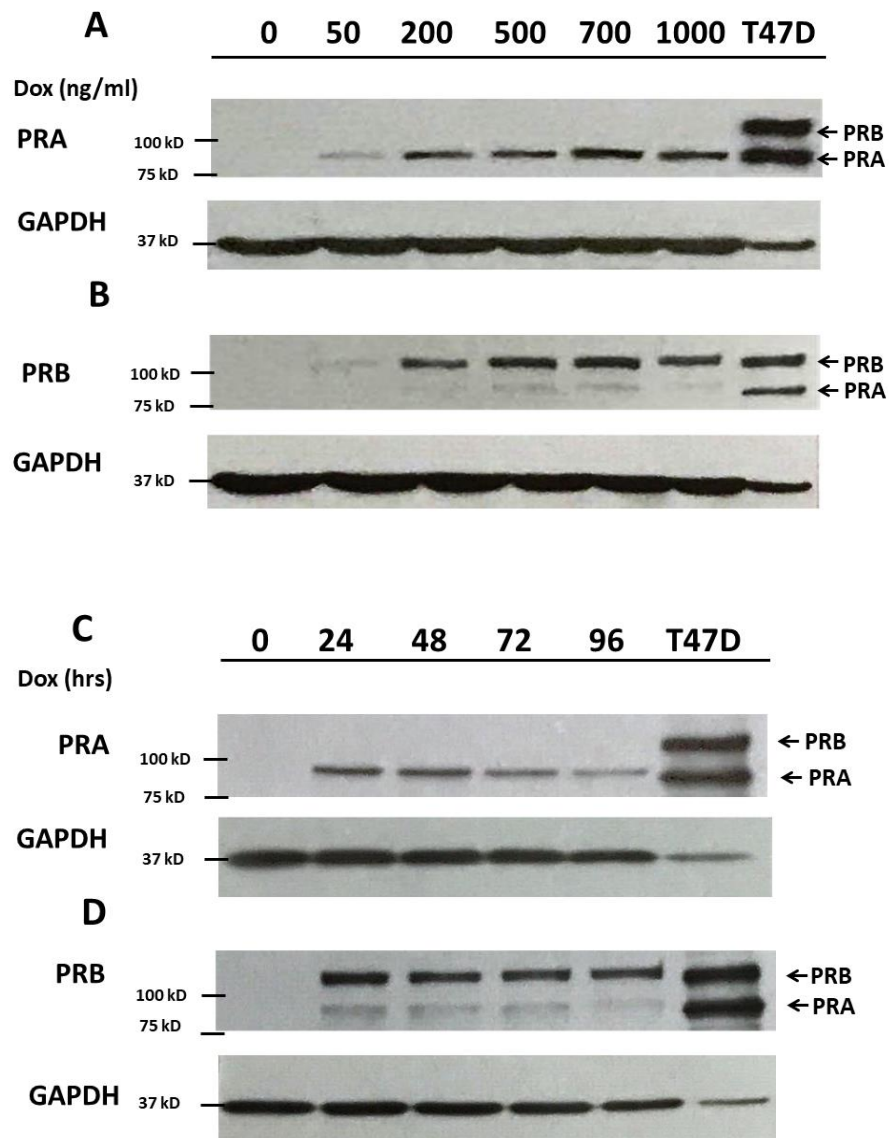
Figure 14 H727-PRA and H727-PRB cell model validation of PRA and PRB protein expression by western blot analysis. (A and B)H727 cells were induced to express PRA and PRB with increasing doxycycline (Dox) concentration (0, 50, 200, 500, 700 and 1000 ng/ml). Dox dose dependently induced PRA (A) and PRB (B) protein expression in H727-PRA and H727-PRB cell model, respectively. (C and D) cells were treated with the most effective dose of Dox (700 ng/ml) in a time course study (0, 24, 48, 72 and 96 hrs) to induce PRA (C) and PRB (D) protein expression, respectively. Western blot analyses were carried out on cell lysates and blots were stained with PR-specific antibody, 1294 mAb. 30μg of protein from H727-PRA or H727-PRB cells were loaded in each lane. 3μg T47D cell lysate was used as positive control. GAPDH was used as loading control.

3. **Characterization of PRB (250H11) monoclonal antibody and validation of tet-inducible PRA and PRB protein localization in H727-PRA and H727-PRB cells model by immunofluorescence.**

To date, most antibodies detecting PR expression detect both PR isoforms, making it difficult to associate PR isoform expression with clinical outcomes in patient samples. To better understand the role of PRB and PRA in lung NET cells, we generated a new PRB specific mouse monoclonal antibody (250H11 mAb). This new PRB-specific (250H11) monoclonal antibody detects an epitope at the N-terminus unique to PRB. Using immunofluorescence staining with 250H11, we showed that PRB (250H11 mAb) antibody detected PR only in H727-PRB cells (Fig15J-15L) but not in H727-PRA cells model (Fig15D-15F) in immunofluorescence.Together, these data indicated that PRB-specific antibody detected only PRB.

Then we examined ectopic PRA and PRB expression in H727 cell model to determine intracellular localization. Cells model were treated with doxycycline 24 hrs and treated with R5020 60 mins. Then we fixed cells and analyzed by immunofluorescence by using PR (1294) and PRB specific (250H11) monoclonal antibody. In H727-PRA cell model without Dox treatment, no PR staining. But with Dox treatment without hormone addition, PR localized mainly in nucleus and when treated with Dox and R5020 (progestin), PR also localized to nucleus. (Fig15A-15C) With the staining of PRB (250H11) specific antibody, there was no PRB expression; mean PRB (250H11) monoclonal antibody is specific (Fig15D-15F). H727-PRB cell model with the staining of PR (1294) (Fig15G-15I) and PRB (250H11) (Fig15J-15L) specific antibody, there were no PR and PRB expression without treatment of Dox. Treatment with Dox in the absence of hormone induced PR and PRB in H727 PRB cell model localized both in nucleus and cytoplasm. However, treatment with Dox plus R5020 caused PR and PRB localized to nucleus. (Fig15I and 15L) PR and PRB staining has the same result as in T47D breast cancer cells but PR has stronger stain than PRB.
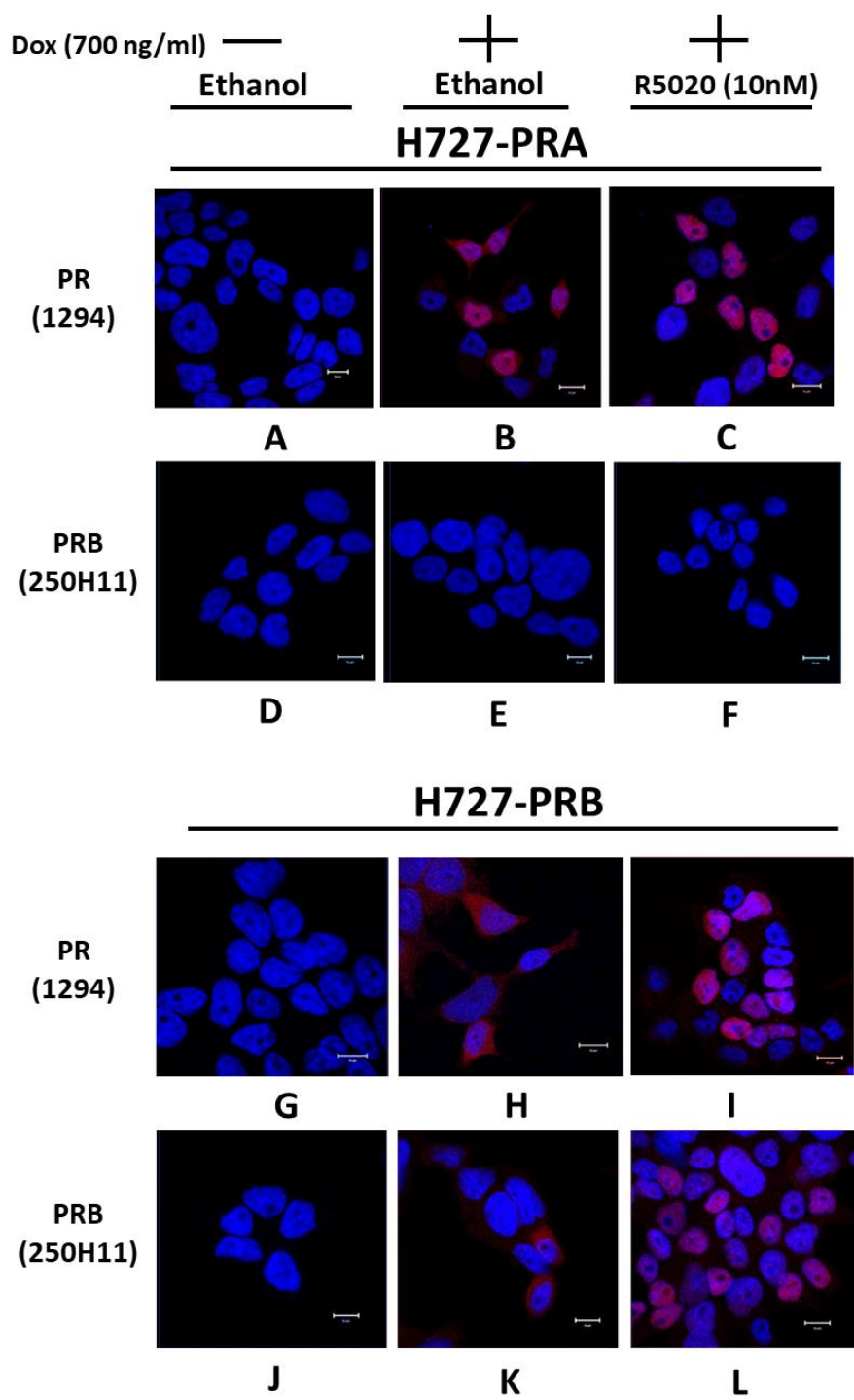
Figure 15 Characterization of PRB-specific (250H11) monoclonal antibody and validated H727-PRA and H727-PRB cells model by immunofluorescence. PR specific (1294 mAb) antibody, (A and G) control untreated cells showed no PR staining. (B and H) H727-PRA and H727-PRB cells treated with 700 ng/ml of doxycycline for 24 hrs, respectively. (C and I) H727-PRA and H727-PRB treated with 700 ng/ml doxycycline for 24 hrs and 10 nM R5020 for 60 min, respectively. PRB specific (250H11) antibody, (D and J) control untreated cells, no PR staining. (E and K) H727-PRA and H727-PRB cells treated with 700 ng/ml of doxycycline for 24 hrs, respectively. (F and L) H727-PRA and H727-PRB cells treated with 700 ng/ml doxycycline for 24 hrs and 10 nM R5020 for 60 min, respectively. Magnification, 1000x, Bars, 10 μm

4.   Validation of tet-inducible PRA and PRB protein transcriptional activity in H727-PRA and H727-PRB cells model by luciferase assay.

For transcriptional activities, H727, H727-PRA and H727-PRB were cotransfected with pRL-CMV (Renilla) and PRETK-LUC plasmid for 24 hours. Cells were controlled protein expression by treated with doxycycline 24 hours. Then H727, H727-PRA and H727-PRB cells were divided into 2 groups of treatment, ethanol (control) and R5020 10 nM for 24 hours. After treatment with Doxycycline and R5020, H727-PRA and H727-PRB were significantly increase luciferase activities compare with ethanol-treated cells. H727-PRB has more transcriptional activities than H727-PRA (Fig16). The differences of PR isoforms transcriptional activities in breast cancer were PRB has more transcriptional activity than PRA (64).
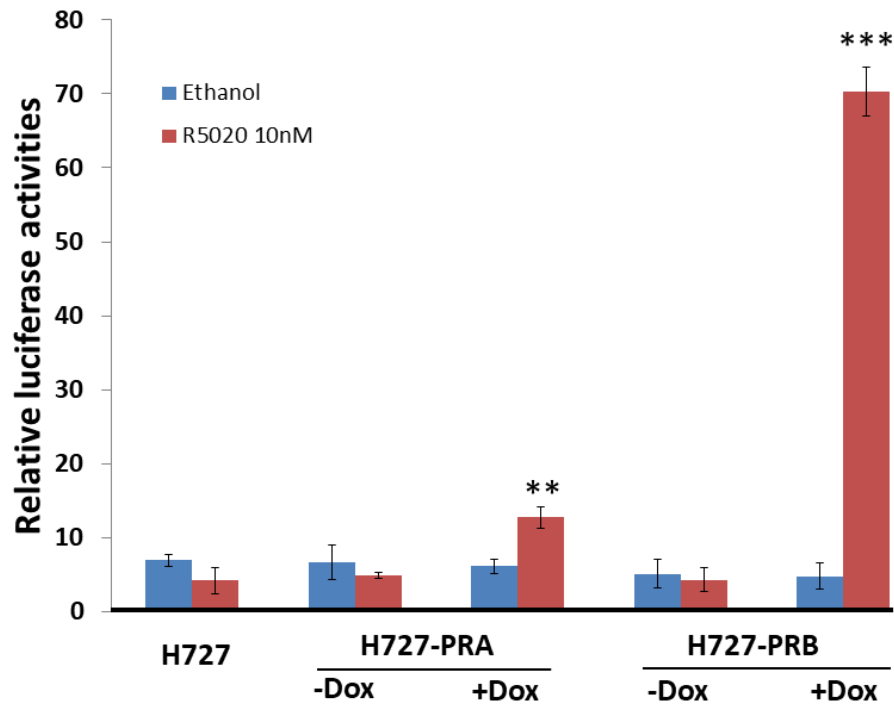
Figure 16 Relative luciferase activities of Firefly and Renilla luciferase of H727-PRA and H727-PRB cell model. Treatment with Dox and R5020 significantly increased luciferase activities in H727-PRA and H727-PRB as compare to cells treated with vehicle (ethanol) (*P≤0.05, **p≤0.01)). Value are represented as means ± SEM from three triplicates (n=3).

5.   Determination of cell viability in H727-PRA and H727-PRB cells model after express PRA and PRB, respectively, by MTT assay.

After cells model validation, we determine cell proliferation. We treated H727, H727 PRA and H727-PRB cell model with Dox in increasing concentration (0, 50, 200, 500, 700 and 1000 ng/ml). We found that at 700 ng/ml of Dox, H727-PRB cell model significantly has lower cell proliferation than H727-PRA cell model and H727 wild type respectively (Fig17). Then we treated cells model with Dox 700 ng/ml for 24, 48 and 72 hrs. H727-PRB also has the lowest cell proliferation than with Dox of H727-PRA and without Dox of H727-PRA and H727-PRB, respectively (Fig18). To compare H727-PRA and H727-PRB cells inhibition, we analyzed percent cell inhibition difference between non PR expression, PRA and PRB expression in H727 cells model. Results show that H727-PRB cells model has higher percent cell inhibition than H727-PRA and H727 without PR expression. (Fig19) However, after treatment with ligands, Dox with vehicle (Ethanol), R5020, RU486 and combination of R5020 and RU486, H727-PRA and H727-PRB cells model have minimal to no effect to the ligands (Fig20A and 20B).
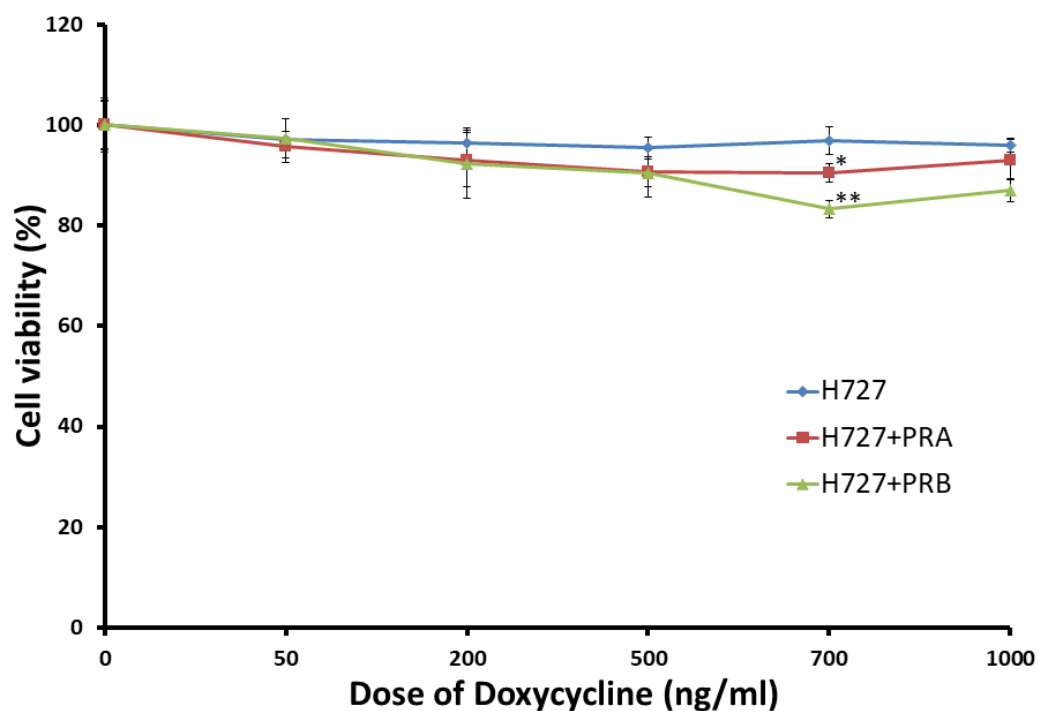
Figure 17 Effects of PRA and PRB on H727 lung NET cell proliferation. H727,
H727-PRA and H727-PRB were treated with increasing concentration of doxycycline
(0, 50, 200, 500, 700 and 1000 ng/ml) for 48 hrs and analyzed by MTT assay. * and
** indicate $p<0.05$ and $p<0.01$ when compared with control untreated cells,
respectively. Values are represented as means ± SEMs from three triplicates (n=3).
Percent cell viability represents % cell viability of doxycycline-treated cells
normalizes to % cell viability of control untreated cells (Doxycycline 0 ng/ml).
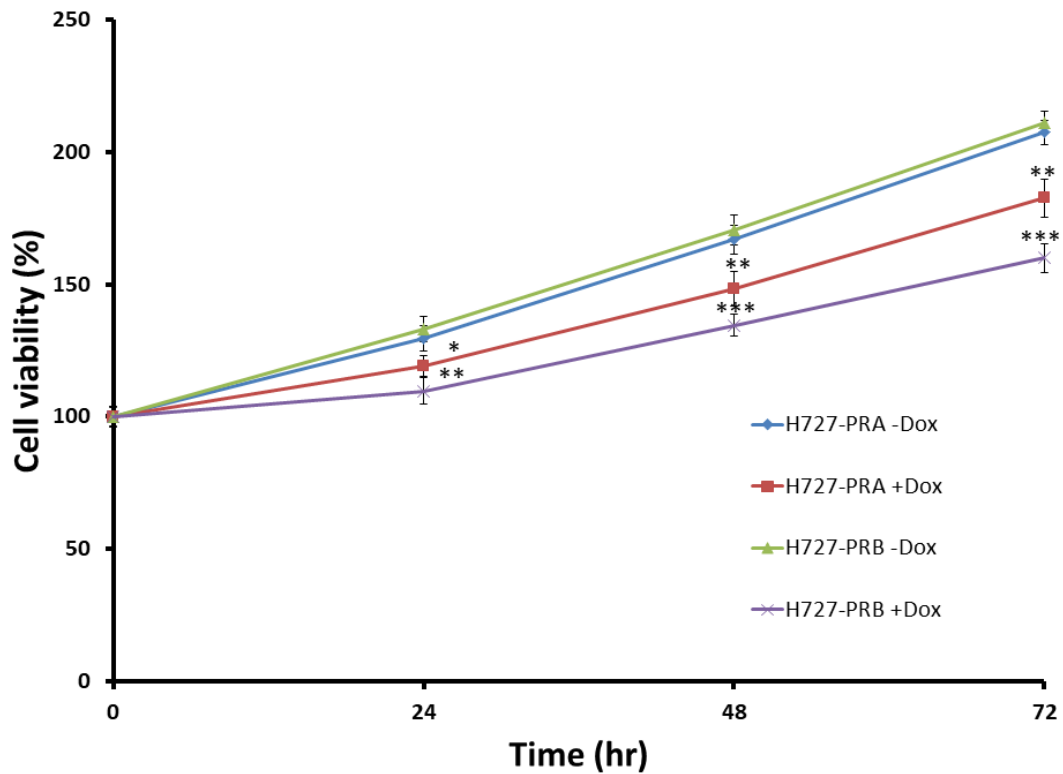
Figure 18 Effects of PRA and PRB on H727 lung NET cell proliferation. H727-PRA and H727-PRB cells were treated with 700 ng/ml Dox and incubated for 24, 48 and 72 hours. ** and *** indicate $p<0.01$ and $p<0.001$ when compared with control untreated cells, respectively. Values are represented as means ± SEMs from three triplicates (n=3). Percent cell viability represents % cell viability of doxycycline-treated cells normalizes to % cell viability of control treated cells (Doxycycline 700 ng/ml, 0 hr).
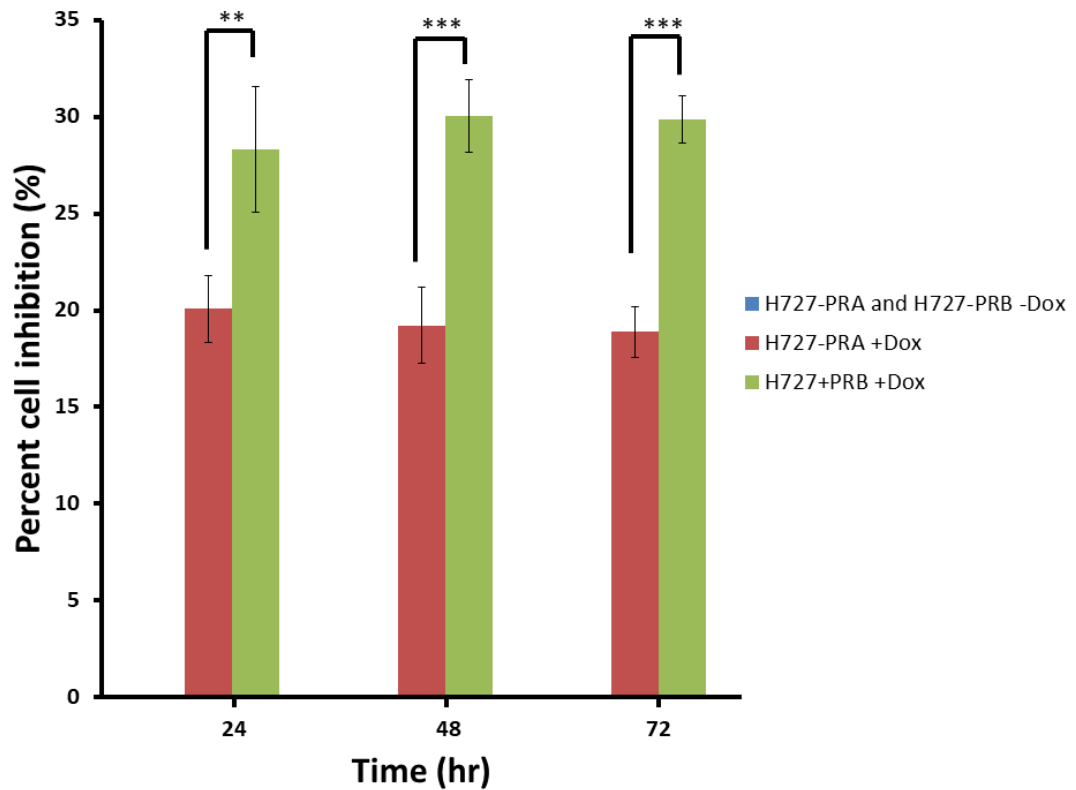
Figure 19 Comparison of percent inhibition of cell growth in H727-PRA and H727-PRB cells. ** and *** indicate p<0.01 and p<0.001 when compared % cell inhibition between H727-PRB and H727-PRA cells. Values are represented as means ± SEMs from three triplicates (n=3). % inhibition by PRA or PRB was calculated by setting control untreated as 100%.
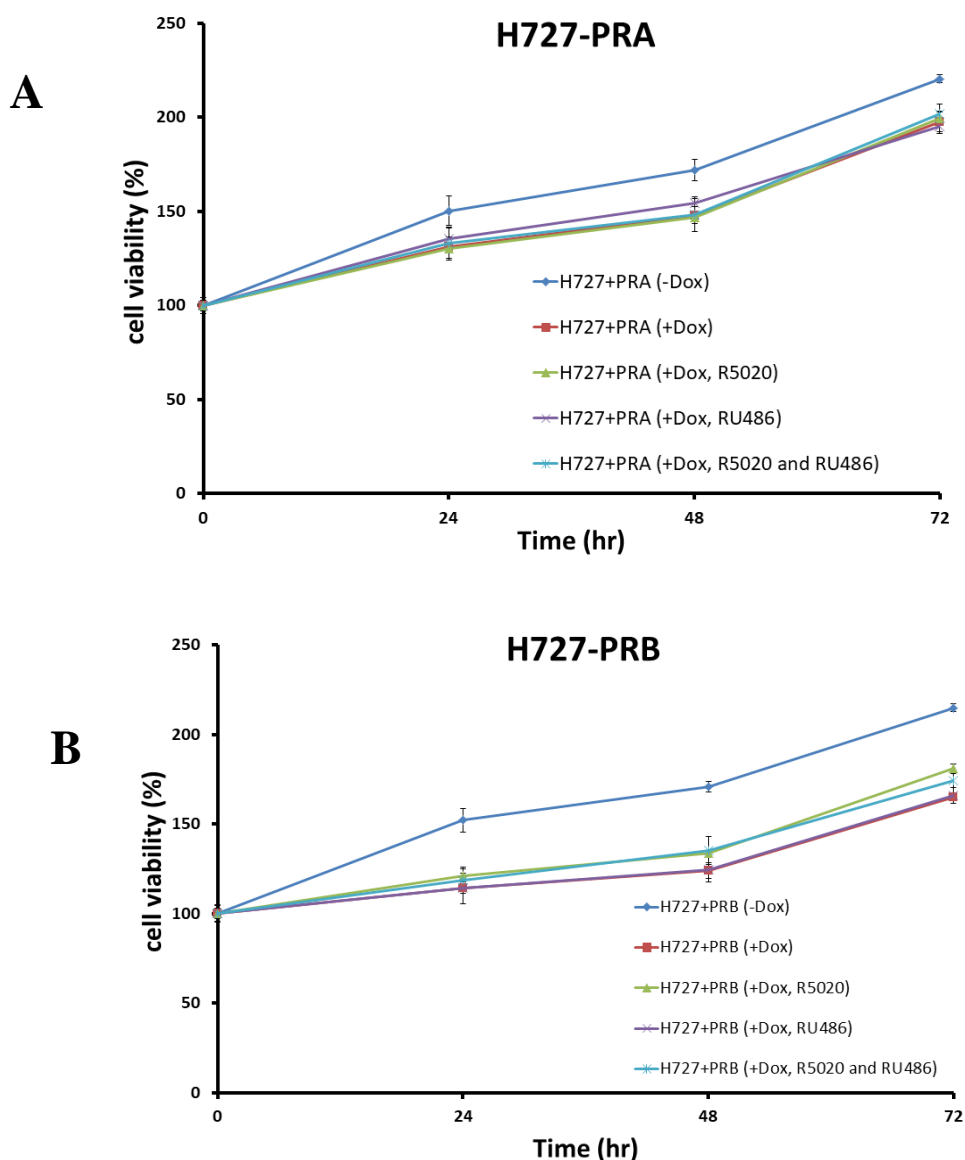
Figure 20 Effects of progestin (R5020) and anti-progestin (RU486) on H727-PRA and H727-PRB cell proliferation. H727-PRA (A) and H727-PRB (B) cells were treated with 700 ng/ml doxycycline, 10 nM R5020, 100 nM RU486 or the combination of 10 nM R5020 and 100 mM RU486 (R5020+RU486). Cells were treated with 700 ng/ml doxycycline for 24 hours then change medium to doxycycline with vehicle (Ethanol), R5020, RU486 and R5020+RU486 and incubated for 24, 48 and 72 hrs. X-axis represents time after R5020 and RU486 treatment. Values are represented as means ± SEMs from three triplicates (n=3). Percent cell viability represents % cell viability of doxycycline-treated cells normalizes to % cell viability of control treated cells (Doxycycline 700 ng/ml and ligands treatment at 0 hr).

6. **Examination of PR isoforms expression in lung neuroendocrine tumors tissues by immunohistochemistry and correlated with clinicopathological factors.**

After we determined cell biological property that PRB expression in H727 cells model cause lower cell proliferation than PRA expression and no PR, we determined PR expression in clinical study to confirm the results. A total of 198 cases of lung NET were examined; 37 (19%) cases were typical carcinoid (TC), 15 (8%) cases were atypical carcinoid (AC), 48 (24%) cases were large cell neuroendocrine carcinoma (LCNEC) and 98 (49%) cases were small cell lung carcinoma (SCLC). Among types of lung NET, carcinoid tumors (TC and AC) were less severe than SCLC and LCNEC. We asked whether PR and PRB proteins express in carcinoid tumor or severe types of lung NET and does it correlate with clinical factors. We did immunohistochemistry in lung NET patients tissues using PR (1294) (Fig21A-21D) and PRB (250H11) (Fig21F-21I) specific antibody and use JMP software to do statistical analysis; Mann Whitney analysis and the $\chi 2$ test. PR and PRB were expressed mostly in TC (Fig21A and 21F) and AC (Fig21B and 21G) and almost absence in SCLC (Fig21C and 21H) and LCNEC (Fig21D and 21I). Moreover, PR and PRB positive cases significantly correlate with lower cell proliferation (Ki-67 and Mitosis) ($p<0.001$). PR and PRB negative cases has Ki-67 approximately 60% and Mitosis/2mm^2 mean 60.PR positive cases has Ki-67 approximately 19% and Mitosis/2mm^2 mean 13 and PRB positive cases has Ki-67 approximately 1% and Mitosis/2mm^2 mean 1. Furthermore, PRB positive cases also have significant lower smoking index and no post operational therapy than PRB negative cases. (Table2)

Clinical variables/ Categories	Total	PR positive H-score > 1%			PRB positive H-score > 1%		
		Negative	Positive	P value	Negative	Positive	P value
Sex							
Male	107(69)	97(91)	10(9)	0.867	100(93)	7(7)	0.667
Female	47(31)	43(91)	4(9)		43(91)	4(9)	
Age, years	68±9	68±8	64±12	0.125	68±9	66±11	0.442
Smoking index	768±626	788±614	578±732	0.234	799±631	383±414	0.034*
WHO classification 2015							
TC	37(19)	30(81)	7(19)		30(81)	7(19)	
AC	15(8)	11(73)	4(27)	<0.001*	11(73)	4(27)	<0.001*
LCNEC	48(24)	47(98)	1(2)		48(100)	0(0)	
SCLC	98(49)	96(98)	2(2)		98(100)	0(0)	
Stages 1/234							
Stage 1	96(64)	85(89)	11(11)	0.233	87(91)	9(9)	0.201
Stage 234	54(36)	51(94)	3(6)		52(96)	2(4)	
Tumor size							
<20 mm	59(39)	51(86)	8(14)	0.140	52(88)	7(12)	0.084
>20 mm	93(61)	87(94)	6(6)		89(96)	4(4)	
Ki-67	58±35	60±33	19±36	<0.001*	61±33	1±2	<0.001*
Mitosis	58±44	60±43	13±26	<0.001*	60±43	1±1	<0.001*
Post-operative therapy							
Yes	56(37)	54(96)	2(4)	0.158	56(100)	0(0)	0.027*
No	95(63)	83(87)	12(13)		84(88)	11(12)	
pTNM Pathological Classification							
pT							
pT 1	92(61)	84(91)	8(9)	0.786	85(92)	7(8)	0.827
pT 23	60(39)	54(90)	6(10)		56(93)	4(7)	
pN							
pN 0	109(76)	97(89)	12(11)	0.356	99(91)	10(9)	0.260
pN 123	34(24)	32(94)	2(6)		33(97)	1(3)	
pM							
pM 0	146(96)	132(90)	14(10)	0.426	135(92)	11(8)	0.485
pM 1	6(4)	6(100)	0(0)		6(100)	0(0)	
Dead/Alive							
Alive	80(56)	70(88)	10(12)	0.247	73(91)	7(9)	0.544
Dead	63(44)	59(94)	4(6)		59(94)	4(6)	
Recurrent Status							
Yes	64(42)	59(92)	5(8)	0.627	61(95)	3(5)	0.310
No	89(58)	80(90)	9(10)		81(91)	8(9)	

Table 2 Association of PR and PRB expression with Clinicopathological factors in Lung NET patients
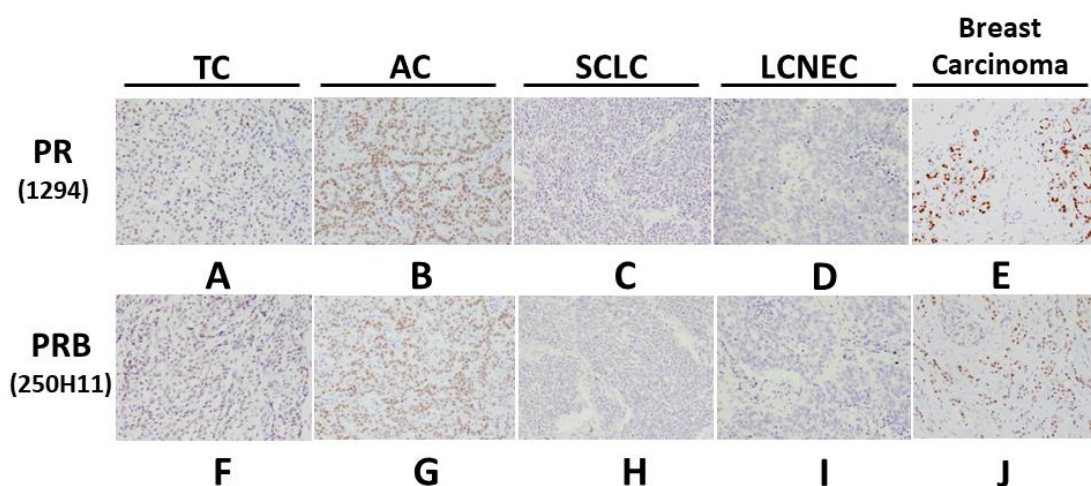
Figure 21 Immunohistochemistry staining of PR (1294) monoclonal antibody (A-D) and PRB (250H11) monoclonal antibody (F-I) in four different lung NET types. Lung NET types including; Typical Carcinoid (TC) (A and F), Atypical Carcinoid (AC) (B and G), Small cell lung carcinoma (SCLC) (C and H) and Large cell neuroendocrine carcinoma (LCNEC) (D and I). Breast carcinoma tissue sections were used as a positive control for PR (E) and PRB (J) staining. (magnification 200x).

7.   Examination of PR isoforms expression in lung neuroendocrine tumors tissues and correlated with survival rates of patients.

Overall survival and disease free survival of lung NET patients were followed up for 10 years (120 months) to determine survival rates associated PR and PRB expression. Comparing with PRB-negative lung NETs, the presence of PRB in lung NETS is significantly associated with longer disease-free survival and lower hazard ratio (Fig 22D). Overall survival of patients with PR (Fig 22A) and PRB (Fig 22C) positive lung NETs and disease-free survival of patients with PR positive lung NETs (Fig 22B) showed a trend for longer survival time than those of patients with PR-negative tumors, but failed to reach statistically significance levels (Table3). Multivariate analysis was performed by Cox-regression test using PR and PRB expression as well as standard pathological variables. The presence of PR and PRB showed a trend to associate with good prognostic markers but failed to reach statistically significance levels (Table 4).
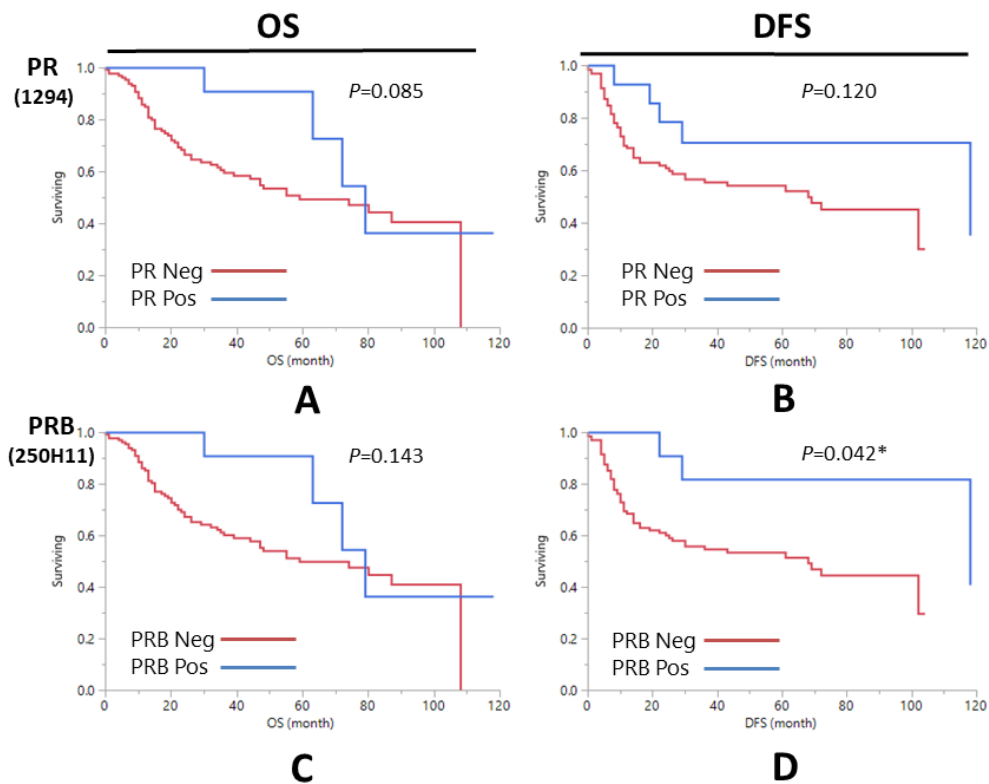
Figure 22 Kaplan-Meier plot of overall survival (OS) and disease free survival (DFS) for all patients by: PR (A and B) and PRB (C and D) status.

Variable	No of patients	No. of Death/ Recurrence	Mean (months)	P-value	Hazard ratio (95%CI)
OS					
PR status negative	139	59	62.73	0.085	1
positive	14	4	70.36		0.41(0.12-1.03)
PR B status negative	142	59	63.23	0.143	1
positive	11	4	70.36		0.47(0.14-1.17)
Sex Male	106	51	57.93	0.009	1
Female	47	12	63.30		0.45(0.23-0.81
Smoking index < 500	49	12	59.17	0.001	1
> 500	96	47	55.57		2.71(1.48-5.36)
WHO classification 2015 TC	37	3	69.64	<0.001	1
AC	15	6	61.38		4.83(1.27-22.92)
LCNEC	29	19	47.94		12.50(4.24-53.30)
SCLC	72	35	44.87		12.07(4.31-50.30)
Stages 1	95	28	77.40	<0.001	1
234	54	35	34.27		3.70(2.23-6.19)
Tumor size < 20 mm	59	16	62.73	0.002	1
> 20 mm	92	47	54.57		2.43(1.40-4.42)
pN 0	108	34	75.24	<0.001	1
123	34	24	28.26		4.26(2.47-7.26)
DFS					
PR status negative	137	57	57.79	0.120	1
positive	14	5	89.22		0.46(0.14-1.12)
PR B status negative	140	59	57.22	0.042	1
positive	11	3	101.18		0.26(0.04-0.84)
Sex Male	105	48	61.73	0.044	1
Female	46	14	71.92		0.55(0.29-0.97)
Smoking index < 500	49	12	79.79	<0.001	1
> 500	94	46	56.29		2.90(1.58-5.76)
WHO classification 2015 TC	37	4	103.66		1
AC	14	6	43.83	<0.001	4.03(1.13-15.94)
LCNEC	29	14	25.79		8.41(2.98-29.96)
SCLC	71	38	31.76		10.12(4.00-34.10)
Stages 1	95	28	82.08	<0.001	1
234	53	31	16.26		3.37(1.99-5.72)
Tumor size < 20 mm	58	19	79.55	0.012	1
> 20 mm	91	42	38.27		2.02(1.17-3.65)
pN 0	108	36	76.49	<0.001	1
123	33	20	15.05		3.50(1.96-6.12)
Abbreviations: OS = Overall Survival; DFS = Disease Free Survival; PR = Progesterone Receptor; PRB = Progesterone Receptor isoform B; pN = Lymph node metastasis; TC = Typical Carcinoid; AC = Atypical Carcinoid; LCNEC = large-cell neuroendocrine carcinoma; SCLC = small-cell lung carcinoma					

Table 3 Summary of OS and DFS analysis

Variable	Hazard Ratio (95% CI)	P-value
OS		
PR status Negative Positive	1 0.42(0.13-1.05)	0.066
PR B status Negative Positive	1 0.50(0.15-1.27)	0.160
Stages 1 234	1 2.57(1.10-5.53)	0.030
Tumor size <20 mm >20 mm	1 1.36(0.75-2.60)	0.317
pN0 123	1 1.78(0.85-4.07)	0.130
DFS		
PR status Negative Positive	1 0.55(0.17-1.36)	0.214
PR B status Negative Positive	1 0.35(0.06-1.14)	0.088
Stages 1 234	1 2.42(0.99-5.36)	0.052
Tumor size <20 mm >20 mm	1 1.24(0.67-2.39)	0.503
pN0 123	1 1.56(0.70-3.79)	0.283
Abbreviations: OS = Overall Survival; DFS = Disease Free Survival; PR = Progesterone Receptor; PRB = Progesterone Receptor isoform B; pN = Lymph node metastasis		

Table 4 Multivariate analysis for OS and DFS

8.   Examination of PR isoforms expression in lung neuroendocrine tumors tissues and correlated with survival rates of patients by separated sex.

Since higher progesterone levels in female could have different effects on PR signaling, we analyzed overall and disease free survival analysis in male and female separately. Although there is no significant correlation of overall survival between lung NET patients with PR and PRB expression as compared to those without PR expression, our analysis suggested that lung NET patients with PR and PRB expression showed a trend for better overall and disease free survival. Interestingly, lung NET female patients whose tumors expressed PR or PRB showed 100% overall and disease free survival, whereas expression of PR or PRB in lung NET male cases showed approximately 50% overall and disease free survival (Table5). There was no significant difference in overall (Fig23A -23D) and disease free survival (Fig24A – 24D) between female and male whose lung NET expressed PR and PRB.

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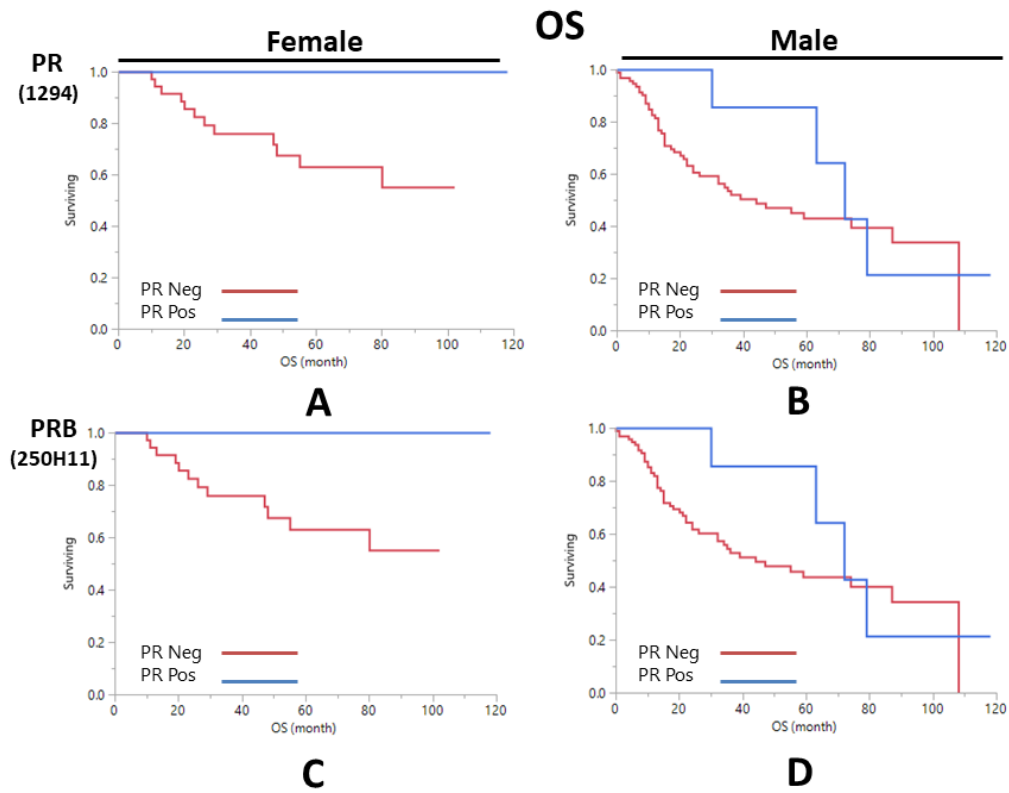
Figure 23 Kaplan-Meier plot of overall survival (OS) separated patients' sex: PR (A) and PRB (C) status in female, PR (B) and PRB (D) status in male.
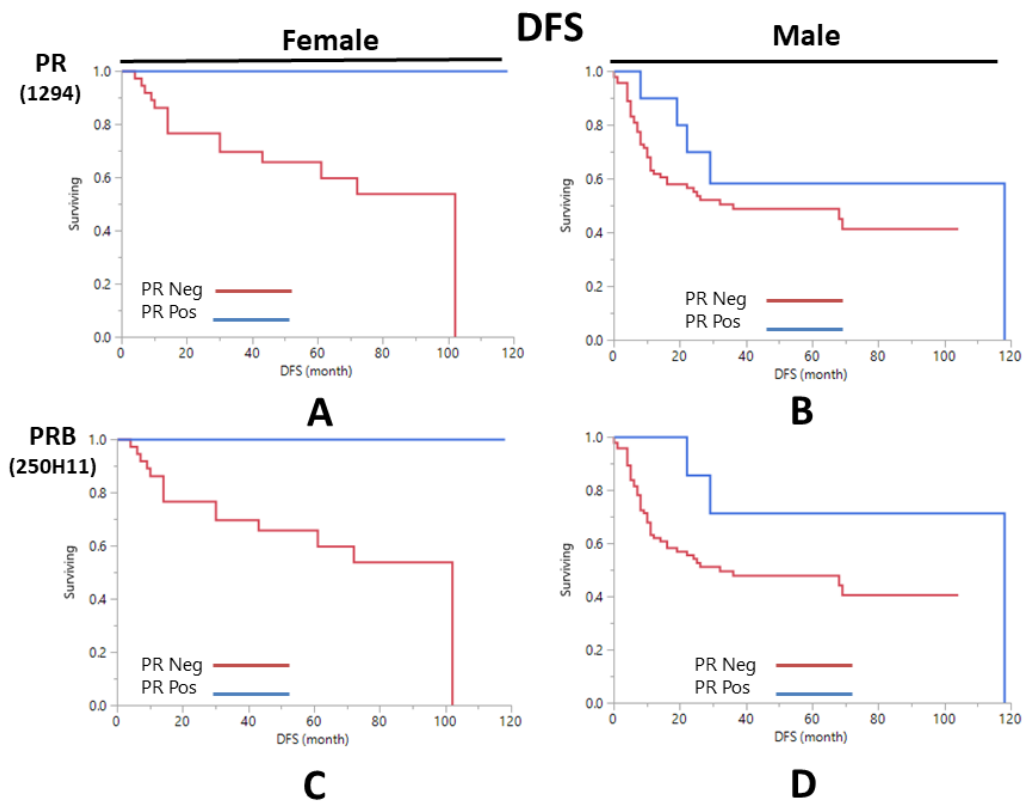
Figure 24 Kaplan-Meier plot of disease free survival (DFS) separated patients' sex: PR (A) and PRB (C) status in female, PR (B) and PRB (D) status in male.

Variable		No of patients	No. of Death/ Recurrence	Mean (months)	P-value
OS					
PR (Male) status negative positive		96 10	47 4	56.78 67.07	0.186
PR (Female) status negative positive		43 4	12 0	61.43 -	0.210
PRB (Male) status negative positive		99 7	47 4	57.60 67.07	0.345
PRB (Female) status negative positive		43 4	12 0	61.43 -	0.210
DFS					
PR (Male) status negative positive		95 10	43 5	39.57 77.12	0.350
PR (Female) status negative positive		42 4	14 0	68.48 -	0.099
PRB (Male) status negative positive		98 7	45 3	39.15 91.57	0.172
PRB (Female) status negative positive		42 4	14 0	68.48 -	0.099
Abbreviations: PR = OS = Overall Survival; DFS = Disease Free Survival; Progesterone Receptor; PRB = Progesterone Receptor isoform B					

Table 5 Summary of PR and PRB status with OS and DFS analysis by separated sex

9. **Examination of PR localization in pulmonary neuroendocrine cells (PNECs) of normal fetal and adult lung by double immunohistochemistry.**

   Airway and alveolar epithelia in the lung contained pulmonary neuroendocrine cells (PNEC) which involve in nerve and endocrine functions. Recent study demonstrated that PR expression in NSCLC is associated with better prognosis (11, 115). However, the role of PR and PR isoform expressions in lung NET is largely unknown. Several evidence suggested that lung NET arises from PNEC (21). In this study, we showed that lung NET, especially TC and AC, expressed PR and PRB. We next examined whether PNEC in normal fetal and adult lung expressed PR or PRB. Fetal and adult lung tissues were cut into three serial sections. The first section was single stained with monoclonal antibody against synaptophysin (SYN) to mark neuroendocrine cells (Fig25A and 25D). The second and third sections were double stained with SYN and PR (1294) (Fig 25B and 25E) or PRB (250H11) mAb (Fig 25C and 25F). There are more SYN positive cells in normal fetal lung than adult lung (Fig25A and 25D). Double staining of SYN & PR and SYN & PRB demonstrated that subset of SYN-positive PNEC expressed PR and PRB. Our results suggested that PR expression in PNEC of fetal lung (Fig25B) more than that of adult lung (Fig25E). PRB expression was detected mostly in fetal lung (Fig25C), and few in adult lung (Fig25F). (Table6) To our knowledge, this is the first report to demonstrate PR and PRB expression in PNEC of fetal and adult lung. More study will be needed to further examine physiological significance of PR and progesterone in PNEC.
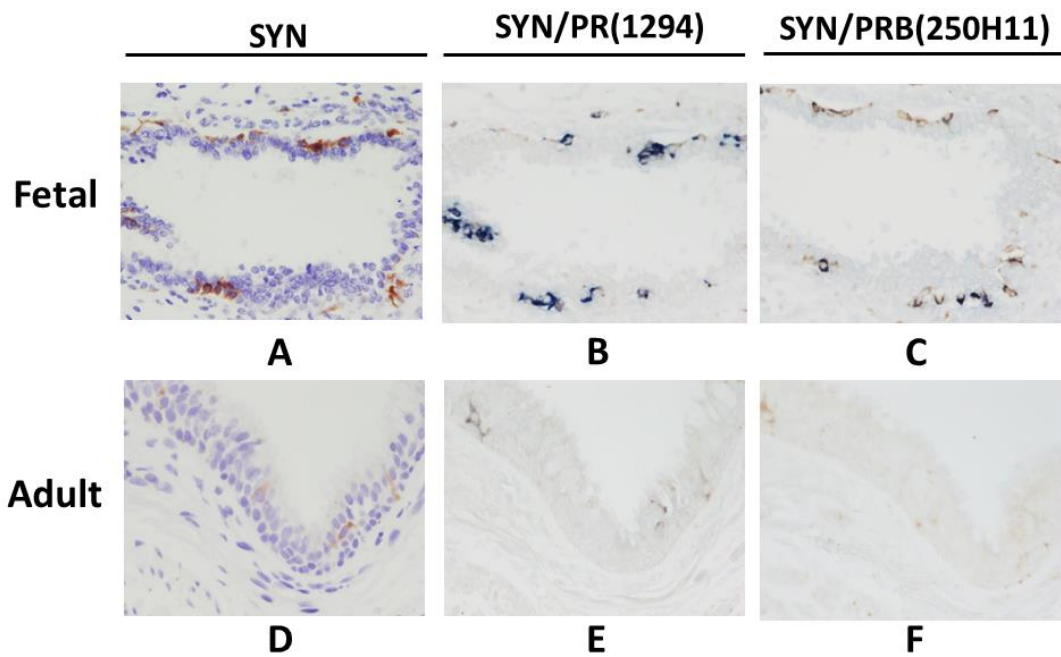
Figure 25 Serial tissue sections of normal fetal and adult lung were stained by immunohistochemistry of SYN and double immunohistochemistry of SYN, PR and PRB. (A and D) tissues were stained with synaptophysin). (B and E) tissues were stained with SYN (brown) in combination with PR (PR, 1294 mAb, blue) and (C and F) were staining of SYN (brown) in combination with PRB specific (250H11 mAb, blue). (magnification 400x).

Normal lung	SYN$^+$: Neg (%)	SYN$^+$PR$^+$: SYN$^+$ (%)	SYN$^+$PRB$^+$: SYN$^+$ (%)
Fetal	2.52±2.86	72.71±10.43	34.33±24.99
Adult	1.18±0.45	58.03±12.68	4.08±7.44

SYN$^+$: Neg = Ratio in percent of SYN immunohistochemistry with negative cells in epithelial bronchi area
SYN$^+$PR$^+$: SYN$^+$ = Ratio in percent of PR and SYN double positive cells with SYN positive cells in double immunohistochemistry
SYN$^+$PRB$^+$: SYN$^+$ = Ratio in percent of PRB and SYN double positive cells with SYN positive cells in double immunohistochemistry

Table 6 Mean of ratio between double IHC of PR and PRB with SYN in Normal fetal and adult lung

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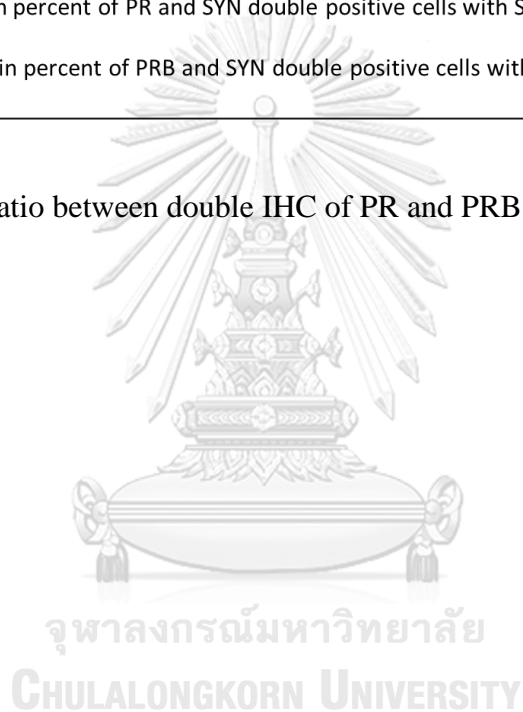
CHAPTER V

DISCUSSION

Several clinical studies showed that PR expression in lung cancer such as NSCLC is correlated with better prognosis. However, the clinical or prognostic values of PR and PR isoforms expression in lung NET are largely unexplored. We examined PR expression in lung NETs *in vitro* by constructing a lung NET cell model. H727 lung carcinoid cell line was used as a cell model for studying roles of PR in lung NET cells. H727 cells used in this study were obtained from ATCC and the identity was confirmed by genomic DNA comparison to the ATCC database. We showed that H727 lacks endogenous PR by real-time PCR and Western blotting. However, internal control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was not shown because of GAPDH expression is different between cell lines which GAPDH is an enzyme involves in glycolytic pathway. GAPDH of T47D CT value was 14 and H727 CT value was 28. We also change to use beta actin (non-muscle cytoskeletal actin) as internal control which still gets about the same result as GAPDH. T47D and H727 actin expression CT value were 27 and 14 respectively. This may because of H727 has lower cell proliferation or cell motility, structure and integrity than T47D cell. Then we constructed H727-PRA and H727-PRB cells model. Tetracycline-inducible PRA and PRB expression in H727 cell lines were constructed to examine how expression of PRA and PRB affected the growth of H727 lung NET cells. We showed that PRA and PRB expressed from this dox-inducible expression system. Treatment of doxycycline 700 ng/ml in 24 hours induced maximum PRA and PRB expression. Moreover, H727-PRA and H727-PRB cells model demonstrated normal PR intracellular localization. PRB was found to be distributed in both nuclear and cytoplasm, while PRA was found to be distributed mainly in the nucleus with limited cytoplasmic in the absence of progestin. Both PRA and PRB demonstrated nuclear translocation upon addition of progestin similar to endogenous PR (67) but the mechanism for PR localization is not well known. Furthermore, we showed that PRA and PRB expressed in H727 were transcriptionally active capable of transactivate PRE-dependent gene expression. Moreover, PRB expressed in H727 was transcriptionally more active than PRA (Fig 12). As shown in previous study, PRA functions as a transcriptional repressor or a weak transcriptional

activator, whereas PRB functions as a transcriptional activator of the same genes in several cell type tested (63, 116).

We used dox-induced expression of PRA or PRB in H727 to directly address how PR expression affected cell proliferation. The growth of dox-induced H727 cell lines expressing PRA or PRB could be directly compared with the growth of same non-induced H727, no PR cell lines. By directly comparing the growth of H727 lung NET expressing PRA and PRB, we found that expression of either PRA or PRB significantly inhibited H727 lung NET cell proliferation as compared to parental PR-null H727 (Fig13). PRB expression significantly inhibited H727 lung NET cell proliferation better than PRA (Fig 13-15). Interestingly, addition of progestin agonist, R5020, and antagonist, RU486 had little to no effect on H727 lung NET cell proliferation, suggesting that PR-mediated inhibition of H727 was dependent on PR expression, independent of ligand. Previous studies also have evidences in ligand-independent PR activation such as in breast cancer(117), PR were phosphorylated of Ser400 by CDK2 could induce PR nuclear localization and transcriptional activity (118). Besides, regulation of PR gene expression by phosphorylated and undersumoylated contribute to breast cancer cell proliferation (119). On the other side, our previously study also demonstrate that PRB expression reduced A549 cells proliferation and interfered with EGFR signaling, in the absence of progestin. Expression of PRB with mutation in the polyproline domain (PPD), ligand for SH3 domain (PR-BΔSH3) failed to inhibit of cell proliferation or interfere with EGFR signaling. Therefore, similar PR extranuclear signaling through PPD interactions could involve in the inhibition of H727 lung NET cell proliferation and interfere with other growth factor signaling (11).

Our *in vitro* study suggested that PR expression inhibited lung NETs cell proliferation and PRB inhibited lung NETs significantly better than PRA. We therefore determined whether results about effects of PR from our *vitro* study could be extended to lung NETs in clinical samples. The association between PR isoforms and clinical outcomes in lung NETs was largely unknown. Previous study suggested that ER/PR expression was not significantly associated with clinical outcome of SCLC and combined SCLC. Either ER and/or PR expression were likely to have shorter survival (8). In this study, we determine the relation of PR expression and

clinical outcomes of all types of lung NETs by using highly specific PR (PRA and PRB) (1294 mAb) and PRB specific (250H11 mAb).

Our new PRB (250H11) specific antibody was highly specific for detection of PRB, but not PRA, as confirmed by immunofluorescence analyses in H727 lung NET cell line expressing individual PR isoform, PRA or PRB (Fig10A and 10B). This new PRB-specific (250H11) monoclonal antibody detects an epitope at the N-terminus unique to PRB while PR (PRA and PRB)-specific (1294) monoclonal antibody detects an epitope at the N-terminus common to both isoforms A and B (91). Unlike previously describe PR isoform specific antibodies (120), our PRB specific 250H11 antibody do not rely on any blocking peptides to confer PRB specificity, making it easy to use in the routine immunohistochemistry examination. Moreover, PRB-specific antibodies; hPRa6 and Ab-6 were validated by immunohistochemistry lack is known in western blot (92, 121). However, some previous show PRB antibody (hPRa6) was done by western blot. Using immunoprecipitation before western blot showed specificity of hPRa6 but using whole lysates for western blot, PRA was detected (93).

Recently, there is no optimal PRA-specific antibody. Moreover, H-score evaluation "PR (PRA + PRB) – PRB = PRA" was not optimal because each staining used different tissue section. Even same patients but different tissue section could show different number of PR or PR isoforms positive cells. Therefore, we could focus only association between PR and PRB expression not PRA in clinical outcome of lung NET patients.

We found that PR and PRB expression were correlated with lower cell proliferation indexes similar to results obtained by our *in vitro* cells model. Among PR positive cases, some cases were found in more aggressive lung NETs types (SCLC and LCNEC). However, PRB expression was only found in less aggressive and more differentiate lung NET types (TC and AC). The presence of PR and PRB show a trend for associations with longer overall and disease free survival. Interestingly, the presence of PRB was significantly associated better disease free survival.

Progesterone level and PR signaling is likely to be different in male and female. Therefore, the presence of PR could have different clinical outcomes in

female as compared to male. We found that approximately 10% of both male and female lung NET patients have PR positive tumors. Yet, the survival rates among PR positives cases were difference in female as compare to male. PR expression in female seemed to be a good prognostic maker and no female lung NET patients with PR positive tumors died within 10-year follow-up time period. While 50% of male lung NET patients with PR positive tumors died during the same 10-year follow-up period. Although there was no statistical significant different between PR positive and PR negative group in male or female due to our small sample size, this dramatically difference in the survival rate between male and female may help us uncover a sex-specific mechanism that could inhibit lung NET proliferation and progression and warrants further analysis in the future .

Several studies reported that lung NET arises from pulmonary neuroendocrine cells (PNEC) which can be found in bronchiolar epithelium of lung (122). In fetus, PNEC are crucial for lung development. PNECs secrete amines and peptide such as synaptophysin or chromagranin A. Whether sex steroid hormones and their receptors such as ER and PR play a role in lung development is not clear. PR was found mainly in reproductive tissues such as in breast and ovarian but recent studies demonstrated that PR could also be found in non-endocrine tissues such as lung. (91) In this study, we demonstrated expression of PR and PRB expression in normal fetal and adult lung. To our knowledge, this was the first report to provide evidence for PR expression in PNEC of both normal fetal and adult lung. PR or PRB double stain with SYN was calculated by counting number of double positive cells but ignore intensity (114). In fetal lung showed very high PR intensity while in adult lung show low intensity. PR expression in PNEC was higher in fetal lung with approximately 72%, high intensity than adult lung with approximately 58%, low intensity. Interestingly, our results suggested that PRB isoform expressed mainly in fetal with approximately 34%, high intensity but few in adult lung with approximately 4%, low intensity. This suggested that PR or PRB expression in PNEC could be involved in lung development and in neuroendocrine system, development of lung and breathing control system. In conclusion, we demonstrated both *in vitro* and in clinical samples that expressions of PR or PRB in lung NET cells are associated with slower cell growth, lower number of proliferating cells and more differentiated Expression of PRB in lung NET is

associated with longer disease free survival. Better understanding of the molecular mechanism of how PR or PR isoform signals in lung NET cells may help us to develop novel therapeutic strategies that will be beneficial for lung NET patients in the future.

จุฬาลงกรณ์มหาวิทยาลัย

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APPENDIX

Experiment data

1. Data of PRA and PRB transcriptional activity in H727, H727-PRA and H727-PRB cells line

Cells Types	Ethanol	10 nM R5020	P value
H727 (-Dox)	0.07±0.008	0.04±0.018	0.071
H727-PRA (-Dox)	0.07±0.024	0.05±0.005	0.278
H727-PRA (+Dox)	0.06±0.009	0.13±0.014	0.002*
H727-PRB (-Dox)	0.05±0.019	0.04±0.015	0.596
H727-PRB (+Dox)	0.05±0.018	0.70±0.034	<0.001*

Data showed firefly/renilla luciferase activity in mean±SD (n=3)

* Significant value $P < 0.05$

2. Comparison of percent cell inhibition in H727-PRA and H727-PRB cells model after treated with doxycycline 700 ng/ml in 24, 48 and 72 hours

Dox (hr)	Percent inhibition of H727-PRA	Percent inhibition of H727-PRB	P-value
24	20.08 ± 1.73	28.33±3.25	0.007*
48	19.22 ± 1.95	30.05±1.88	0.001*
72	17.78 ± 2.43	26.99±1.52	0.005*

Data showed percent cell inhibition in mean±SD (n=3)

* Significant value   P < 0.05

APPENDIX B
Reagents preparation

Preparation of culture medium DMEM

RPMI	47	ml
FBS	2.5	ml
Penicilin streptomycin	500	µl

Preparation of culture medium RPMI

RPMI	44.5	ml
FBS	5	ml
Penicilin streptomycin	500	µl

Preparation of MTT solution 5mg/ml

MTT	250	mg
PBS	50	ml

Filtered by sterile filter PVDF membrane, size 0.22 µM. Then stored at 20 °C in dark.

Preparation of 4% leamli and 10%BME

0.5 M Tric pH 6.8	12.5	ml
SDS	2	g
Glycerol	10	ml
Bromphenol Blue	5	mg
BME	5	ml

Preparation of Sodium Dedecyl Sulfate Poly-Acylamide Gel Electrophoresis (SDS-PAGE gel)

6% stacking gel

MilliQ water	5.2	ml
30% acrylamide	2	ml
0.5 M Tric pH 6.8	2.5	ml
10% SDS	100	µl
10% APS	100	µl
TEMED	10	µl

10% separation gel

MiliQ water	3.2	ml
30% acrylamide	2.67	ml
1.5 M Tris pH 8.8	2	ml
10% SDS	80	µl
10% APS	80	µl
TEMED	8	µl

For gel polymerization, 10% APS and TEMED were added in final step. Glass plates were put on both sides in gel caster. Separation gel was filled between 2 glass plate water was filled for disposing of bubbles. Gel caster was left until separation gel was polymerized. Then removed and filled stacking gel until full. Then a comb was put in. After the gel was polymerized, the comb was removed. The gel which coupled by mirrors was soaked inside 1x Running buffer (10x running buffer 100 ml, 20%SDS 5 ml, milliQ water 900 ml) and prepared for electrophoresis.

Preparation of chemical for Western blot
10x running buffer

Tris	30	g
Glycine	144	g
milliQ water	1000	ml

1x Running buffer

10x running buffer	100	ml
20% SDS	5	ml
milliQ water	900	ml

Transfer buffer

10x running buffer	100	ml
Methanol	100	ml
20% SDS	500	µl
milliQ water	800	ml

TBST buffer

Tris base	20	ml
NaCl	30	ml

| Tween 20 | 500 | μl |
| milliQ water | 950 | ml |

5% BSA for preparation of primary antibody and secondary antibody

| BSA | 0.5 | g |
| TBST | 10 | ml |

จุฬาลงกรณ์มหาวิทยาลัย

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VITA

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