博士論文

Expression of PRMT5 in lung adenocarcinoma and its significance in epithelial mesenchymal transition

「原発性肺腺癌におけるPRMT5の発現パターンと上皮間葉系転換との関連性について」

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Abbreviations

ATS	American Thoracic Society
ARID1A	AT Rich Interactive Domain 1A
BRG1	Known as SMARCA4, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4
BRM	Known as SMARCA2, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
BSA	Bovine serum albumin
C	Cytoplasmic
CK7	Cytokeratin 7
DAB	3,3’-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DR4	Death receptor 4
DR5	Death receptor 5
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition

ERS	European Respiratory Society
ES cells	Embryonic stem cells
EZH2	Enhancer of zeste homolog 2
FGFR1	Fibroblast growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
H3R8	Histone H3 arginine 8
H4R3	Histone H3 arginin 3
IASLC	International Association for the Study of Lung Cancer
MET	Met proto-oncogene
MLL	Myeloid/Lymphoid Or Mixed-Lineage Leukemia
MUCI	Mucin 1, cell surface associated
N	Nuclear
NSCLC	Non-small cell lung cancer
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with Tween 20
PRMT5	Protein arginine methyltransferase 5

P44	Androgen receptor (AR)-interacting protein
SCLC	Small cell lung cancer
SWI/SNF	SWItch/Sucrose NonFermentable
TMA	Tissue microarray
TRU	Terminal respiratory unit
TTF-1	Thyroid transcription factor 1
ZEB1	Zinc finger E-box binding homeobox 1

Chapter 1. Introduction

Section 1.1 Lung cancer

Lung cancer is considered to be a leading cause of cancer death in many developed countries, including the United States and Japan. The estimated number of deaths is around 1.4 million deaths each year. Each year around 1.6 million new cases is diagnosed throughout the world, with estimated 5 year survival rate of about 15% [(1)].One of the major reasons for poor survival is the metastatic potential of the disease and the lack of systematic treatment to deal with these metastatic cancers. Lung cancer is divided into two main types, (i) small cell lung cancer (SCLS) and (ii) non-small cell lung cancer (NSCLC).Small cell lung cancers represents around 20% and the other 80% is non-small cell lung cancers [(2)]. The non-small lung cancer is further subdivided into three histopathological subtypes, (i) squamous cell carcinoma (25%) (ii) adenocarcinoma (40%) (iii) large cell lung carcinoma (15%) [(1)]. Adenocarcinoma is the most common histopathological subtype of primary lung cancer and is a major focus of research to improve patient survival [(3)], and the proportion of adenocarcinomas has increased over the last decades rendering it the most common subtype at the present time [(4)]. Recently a new classification of adenocarcinoma has been published; it was developed by a joint working group of the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS).The details of this classification is shown in Table 1 [(5)]. Finally their work result in confirming that the new subtypes of adenocarcinoma in situ, minimally invasive adenocarcinoma and lepidic-predominant adenocarcinoma had a 5-year survival approaching 100%, whereas micropapillary-predominant and solid with mucin-predominant adenocarcinomas were associated with particularly poor survival. Papillary-predominant and acinar-predominant adenocarcinomas had an intermediate

prognosis [5]. From the entire above facts I believe that lung cancer study and especially lung adenocarcinoma, as the most common subtype, is mandatory to improve the patient survival by searching for new molecular therapeutic targets.

Section 1.2 Epidermal growth factor receptor (EGFR) inhibitors and EMT in lung adenocarcinoma

The most widespread use of epidermal growth factor receptor (EGFR) inhibitors is against NSCLC, [6] as EGFR mutation is known to be associated with lung adenocarcinoma [7]. Epidermal growth factor receptor (EGFR) inhibitors, have a certain therapeutic efficacy for lung cancer patients, however all patients who develop metastatic lung cancers eventually develop resistance to EGFR inhibitors [8]. Although EGFR mutation is one of the mechanisms of resistance acquisition, however the histologic transformation such as epithelial-mesenchymal transition (EMT) is known to cause resistance to EGFR inhibitors in lung adenocarcinoma independent of a T790M mutation which is the most famous mutation of EGFR [8].

EMT is a phenomenon of epithelial cells to lose epithelial phenotype and to gain mesenchymal phenotype temporally or permanently, which is correlated with embryology, wound healing, invasion or metastasis of cancer, and acquisition of drug resistance [9]. Loss of E-cadherin, as a gold standard of EMT, was seen in about 10% of resected primary lung adenocarcinoma cases [10]. EGFR mutations were frequently seen in well to moderately differentiated adenocarcinoma cases with lepidic growth pattern, while lung adenocarcinoma cases with loss of E-cadherin less frequently harbored EGFR mutations [11]. So far there is no

effective therapeutic method is established for lung cancer with features of epithelial-mesenchymal transition (EMT), in which EGFR mutations are less frequent [11].

Section 1.3 Research background

My research is depending on our previous finding which demonstrated that lung adenocarcinoma could be classified into two groups based on the patterns of gene expression and genetic abnormalities; bronchial epithelial phenotype tumors and mesenchyme-like phenotype tumors [7]. "Bronchial epithelial phenotype" represents a group of lung adenocarcinomas with high expression of bronchial epithelial markers. This group includes thyroid transcription factor (TTF)-1 positive terminal respiratory unit (TRU) type [12] in addition to TTF-1 negative tumors with high expression of bronchial epithelial markers such as CK7 and MUC1 [7]. Bronchial epithelial phenotype tumor exhibits high phosphorylation of EGFR and MET and frequent mutations or amplifications of EGFR, MET, and HER2. In contrast, mesenchymal-like phenotype tumors were characterized by the absence of the bronchial epithelial markers, triple-negative for TTF-1, MUC1, and CK7, showed no or little phosphorylation of EGFR and MET, no mutation or amplification of EGFR, MET, or HER2, and with features of epithelial mesenchymal transition (EMT), such as low E-cadherin and high FGFR1, vimentin, and ZEB1 expressions [7]. The absence of mutations or amplifications of EGFR, MET, or HER2 in mesenchyme-like phenotype tumors suggested that other genetic or epigenetic abnormalities may play a role in this group of tumors.

In recent years, identification of mutations of epigenetic regulatory factors such as MLL, EZH2, ARID1A, DNMT3a...etc. in various tumors has been a hot topic [13] [14] [15] [16].

Interestingly, tumors with these mutations showed undifferentiated, stem cell-like, and EMT phenotype, which suggests that epigenectic mechanisms will be correlated with EMT of tumors. In other report we revealed that loss of BRG1 and BRM, two core catalytic units of SWI/SNF chromatin remodeling complexes, was correlated with tumor formation with EMT features [(10)].

In this study, we tested the expression of histone methyltransferases and demethylases in lung adenocarcinoma taking into account the two groups; bronchial epithelial phenotype and mesenchymal-like phenotype, mentioned previously. We focused on PRMT5 as the best candidate according to our selection criterion (see Methods and Results sections).

Section 1.4 PRMT5 (Protein arginine methyltransferase 5)

PRMT5 is a member of the protein arginine methyltransferase family. These enzymes catalyze the methylation of arginine residues on histone and non-histone substrates. PRMT5 belongs to type II subgroup which catalyzes the symmetrical dimethylation of arginine in histone and non-histone substrates. It plays multiple roles in cellular processes, including cellular differentiation [(17) (18); (19)], cellular proliferation [(20)], apoptosis [(21)], ribosome biogenesis [(22)], assembly of the Golgi apparatus [(23)], etc. Overexpression of PRMT5 is found in many cancers, including gastric cancer [(24)], leukemia and lymphoma [(25) (26)], breast cancer [(27)], prostate cancer [(28)], and colorectal cancer [(21)] and is related to poor outcome in patients [(27); (21)]. PRMT5 localizes to both nucleus and cytoplasm, in the nucleus it methylates histone H3R8 and histone H4R3 and trigger transcriptional silencing. In cytoplasm it methylates many targets such as EGFR, DR4,DR5, Sm proteins,…etc [(29)]. However, little is known about the functional difference between nuclear and cytoplasmic PRMT5 in lung adenocarcinoma.

In the present study I tested the expression of PRMT5 in lung adenocarcinoma aiming to identify the difference in expression pattern in various types of cell lines (bronchial epithelial and mesenchymal-like phenotypes), as well as in different histopathological subtypes of lung adenocarcinoma using primary tissue samples. I also described the distinct expression patterns of PRMT5 in terms of cytoplasmic and nuclear localization and its significance in malignant progression, especially in EMT.

Although overexpression of PRMT5 was reported in lung cancer [30], the expression pattern in terms of cytoplasmic and nuclear localization in various histopathological subtypes of lung adenocarcinoma and its relation to bronchial epithelial markers (TTF-1, CK7, MUC1, and E-cadherin), genetic alterations such as EGFR mutation, clinicopathological factors, and patient survival have not been studied yet. So I studied these relations in details.

Chapter 2. Materials and Methods

Section 2.1 Gene expression profile of 40 Non-small cell Lung carcinoma cell lines

I used microarray analysis data of 40 non-small cell lung carcinoma cell lines; 35 adenocarcinoma cell lines, 4 large cell carcinoma cell lines, and one adenosquamous cell line. A comprehensive gene expression analysis was performed using an oligonucleotide microarray (GeneChip Human Genome U133A, Affymetrix, Santa Clara, CA) as described previously [(4)].

Section 2.2 Cell lines and Medium

All cell lines were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% antibiotics in a humidified atmosphere with 5% CO2 and 95% air.

I used microarray analysis data of 40 non-small cell lung cancer cell lines; 35 adenocarcinoma cell lines (H23, H292, H358, H441, H522, H596, H650, H1395, H1648, H1650, H1651, H1703, H1781, H1793, H1838, H1975, H1993, H2009, H2087, H2228, H2405, HCC827, HCC4006, Calu-3, A549, ABC-1, PC3, VMRC-LCD, RELF-LC-Ad1, RELF-LC-Ad2, HLC-1, LC-2/ad, RERF-LC-KJ, and L27), 4 large cell carcinoma cell lines (Lu65, H460, H661, and H1299), and one adenosquamous cell line (H596). In our previous report, we demonstrated that 40 lung carcinoma cell lines could be classified into two groups; bronchial epithelial phenotype (n=22) and mesenchyme-like phenotype (n=18) [(7)]. Here I used A549, H522, H1651, HCC4006, H1650, and PC3 for immunocytochemistry. HCC4006, H1650, and PC3, three EGFR-mutated cell lines, are representatives of bronchial epithelial phenotype, and A549, H522, and H1651 are representatives of mesenchyme-like phenotype [(7)].

Section 2.3 Immunocytochemistry

I used the following adenocarcinoma cell lines A549, H522, H1651, HCC4006, H1650, and PC3. HCC4006, H1650, and PC3 are bronchial epithelial phenotype, A549, H522, and H1651 are mesenchyme-like phenotype. Chamber slides of 4 wells were used (Sigma).Cells were seeded on chamber slides, and then washed by PBS two times. Fixation was done using 4% paraformaldehyde for 10 minutes at room temperature. Followed by three-5 minutes PBS washes with gentle shaking. For permialization, triton X 0.05% was used for 5 minutes at room temperature followed by Three-5 minutes PBS washes with gentle shaking. Blocking was done by incubation at room temperature with 3% BSA for 10 minutes. Then the antibody (anti-PRMT5, rabbit polyclonal, HPA005525 Sigma, 1:50) was incubated with the cells overnight at 4^0 C.

The following day, the cells were washed three times with PBS followed by incubation with second antibody (anti-rabbit Alexa Flour 488, Invitrogen) for one hour at room temperature. Then washing with PBS for three times and finally mounting with mounting medium with DAPI. Then the slides were observed under fluorescence microscope.

Section 2.4 Western blotting

I used the adenocarcinoma cell lines A549, H522, H1651, HCC4006, H1650, and PC3. Cells were grown to confluence and then collected and centrifuged for 5000 RPM for 5 minutes. Next the cells were lysed with a lysis buffer (50 mM Tris PH 7.8, 150 mM NaCl, 1mM EDTA, and 1% Triton X-100). The cells were incubated on ice for 10 minutes, and centrifuged in 15,000 for 15 minutes. The supernatants were collected into new tubes as the whole cell lysates. The protein concentration of each sample was determined using Bio-Rad Protein assay according to

manufacturer protocol. Samples were denatured and 10 ug of protein were applied to acrylamide gel followed by electrophoresis, and then transferred into PVDF membrane using the semi-dry method. The membrane was blocked in 5% skimmed milk solution for 1 hour at room temperature, then incubated with the specific antibodies ; PRMT5 (rabbit polyclonal, Sigma, 1:500), vimentin (mouse, BD biosciences, 1:2000), E-Cadherin (mouse, BD biosciences, 1:2500), TTF-1 (mouse monoclonal, clone 8G7G3/1 Dako 1:500), CK7 (mouse monoclonal, clone OV-TL 12/30 Dako 1:500), and MUC1 (hamster monoclonal, MUC1 smaller cytoplasmic subunit Lab vision1:250), overnight at 4^0C. Then washing the membranes from the first antibody with PBST for 3 times was done. Then the membrane was incubated for one hour at room temperature with second antibodies; Anti-rabbit IgG peroxidase conjugate (Amersham), Anti-mouse IgG peroxidase conjugate (Amersham), and Anti-Armenian hamster IgG peroxidase conjugate (Jackson Immunoresearch). Followed by three times wash with PBST. Finally the protein bands were visualized using enhanced chemiluminescence with a protein-blotting detection (GE Healthcare).

Section 2.5 Tissue microarray sections

I used 7 different TMAs, which were produced to accommodate primary lung adenocarcinoma tissue core sections, for immunohistochemistry. These samples were collected from patients (n=166) who had undergone surgical resection at the University of Tokyo Hospital between June 2005 and September 2008. Informed consent was obtained from all the patients, and the study was approved by the Institutional Ethics Review Committee. Of 166 core sections, 22 were missing from TMA sections, and 14 invasive adenocarcinoma cases showed only lepidic

growth components without invasive lesion on TMA sections. The patients (n=130) included 75 males and 55 females, ranging in age from 34 to 86 years (average 66.8 years). Each case was reassigned for tumor, node, metastasis (TNM) classification and pathological stage on the basis of the new IASLC staging system (31); 85 were Stage I (52 Stage IA, 33 Stage IB), 15 were Stage II (12 Stage IIA, 3 Stage IIB), 23 were Stage III (18 Stage IIIA, 5 Stage IIIB), and 2 were stage IV. Stages of 5 cases were unknown (3 were more than Stage I). 130 cases included 17 non-mucinous adenocarcinoma in situ and 113 invasive adenocarcinomas. Each case was classified by pathologist (D.M) as follows, depending on predominant histopathological subtype in invasive lesion on the TMA sections; lepidic growth components (without invasive lesion) (n=17), papillary adenocarcinoma (n=56), acinar adenocarcinoma (n=25), solid adenocarcinoma with mucin (n=29), and invasive mucinous adenocarcinoma (n=3). These cases were also graded into three grades; low-grade containing lepidic growth (n=17), intermediate-grade containing acinar and papillary adenocarcinomas (n=81), and high-grade containing solid adenocarcinoma with mucin and invasive mucinous adenocarcinoma (n=32), referring to the histopathological grading by Yoshizawa et al [(5)] with a slight modification.

Section 2.6 Immunohistochemistry

I performed immunohistochemistry using a tissue microarray that included the samples. Tissue microarray sections were deparaffinized in three 5-minute washes of 100% xylene, followed by hydration in four 5-minutes washes of graded ethanol. Antigen was retrieved by autoclaving using HISTOFINE PH9 (Dako) for 10 minutes at 120^0 C. Then endogenous peroxidase was blocked using 0.3% hydrogen peroxide with methanol for 10 minutes, followed by applying of 2% BSA to reduce nonspecific binding for 10 minutes. Then antibodies were

applied to the TMA sections and incubated overnight at 4^0 C; PRMT5 (rabbit polyclonal, HPA005525 Sigma, 1:200), TTF-1 (mouse monoclonal, clone 8G7G3/1 Dako, 1:100), CK7 (mouse monoclonal, clone OV-TL 12/30 Dako, 1:100), MUC1 (mouse monoclonal, clone MA695 Novocastra, 1:100), and E-cadherin (mouse monoclonal, clone 36 BD Biosciences, 1:400). The following day, TMA sections were washed with PBST and incubated with EnVision labelled polymer peroxidase (Dako) for 30 minutes. Subsequently, tissue microarray sections were incubated with DAB+chromogen (Dako) for 3 minutes for detection of antigen. The DAB reaction was stopped by applying MQ-H2O and then sections were counterstained by hematoxylin staining to locate the nucleus. All steps were held at room temperature except for the specified temperatures. The slides were examined under the light microscope.

Section 2.7 Evaluation of Immunohistochemistry

The evaluation of the immunohistochemical staining was performed by two pathologists (D.M, and A.G.), independently, through light microscopic observation and without knowledge of the clinical data from each patient. Cases of disagreement were reviewed jointly to reach a consensus score. PRMT5 immunoreactivities were evaluated in nucleus and cytoplasm respectively. Intensity was quantified as follows: (+) means a staining was detected and hence expression was detected and (-) means no or weak staining was detected. MUC1, TTF-1, CK7, and E-cadherin immunoreactivities were evaluated in accordance with the methods in previous report (4). In brief, nuclear staining was assessed for TTF-1, while membranous and cytoplasmic staining was assessed for CK7 and E-cadherin. Membranous staining was assessed for MUC1 (membranous), and expression observed over the entire tumor cell surface was assessed for

MUC1 (depolarized). Immunoreactivity was evaluated semiquantitatively based on the intensity and estimated percentage of tumor cells that were stained.

Section 2.8 Bioinformatic Analyses

Cluster program (*http://rana.lbl.gov/EisenSoftware.htm*) for cluster analysis of the gene expression data of cell lines was used. In brief, we performed average linkage hierarchical clustering of 40 cell lines, using median centering, mean centering and normalization of genes. Next we displayed the results with the aid of TreeView software (*http://rana.lbl.gov/EisenSoftware.htm*, last accessed on March 21, 2008). The image used a color code to represent relative expression levels. Red represents expression levels greater than the mean for a given gene across all samples. Green represents expression levels less than the mean across samples.

Section 2.9 Statistics

The chi-squared test was used to evaluate the clinicopathologic correlations except histopathological grades. Mann-Whitney U-test was used to evaluate the histopathological grades, with each grade scored (low-grade 1. intermediate-grade 2. and high-grade 3.). Overall Survival curves were generated using the Kaplan–Meyer method and differences in survival were analyzed by the Wilcoxon method. The results were considered significant if the p value was less than 0.05 (Bonferroni correction was used in multiple comparisons). All statistical

calculations were performed using the StatView computer program (Abacus Concepts, Berkeley, USA).

Chapter 3. Results

Section 3.1 PRMT5, a candidate gene involved in EMT, among histone methyltransferases and demethylases, depending on oligonucleotide array analysis of 40 cell lines

In my search for histone methyltranseferases and demethylases correlated with EMT. First, I extracted expression profile data for the histone methyltransferases and demethylases and examined the relative expression levels of these genes in the 40 lung cancer cell lines. Next, I calculated the correlation coefficients of vimentin and E-cadherin for each gene, and selected the genes which met the following requirement (Correlation coefficient with vimentin – correlation coefficient with E-cadherin) x ½, the significance level of correlation coefficient was set as > 0.3, or <-0.3. The results are shown in Table 2. I used vimentin and E-Cadherin because they are the most famous markers in EMT, usually tumors with EMT features shows high vimentin and low E-Cadherin expression. Then I chose PRMT5 as the best suitable candidate correlated with EMT. Then hierarchical cluster analysis of 40 lung cancer cell lines was performed, based on the gene expressions of PRMT5, TTF-1, MUC1, CK7, E-cadherin, and vimentin. I found that PRMT5 was really correlated with vimentin and frequently highly expressed in vimentin high cell lines (Fig. 1). A549 cell line is included in the group of cells that expresses high levels of E-cadherin. The justification of this is as follows: In our previous study, we performed cluster analysis using many genes associated with cancer invasion and proliferation, considering the genetic status of EGFR, KRAS, MET, and HER2 and activation of EGFR and MET, then we classified A549 into mesenchymal-phenotype. On the other hand, Figure 1 just shows the cluster analysis depending on only 6 markers (E-cadherin, TTF1, CK7, MUC1, VIM, and PRMT5). Therefore this cluster analysis was not absolute but only shows the tendency; that is, PRMT5 was highly expressed in cell lines with high levels of vimentin and low levels of E-cadherin and the other bronchial epithelial markers (TTF-1, CK7, and MUC1). As A549 is showing high levels of vimentin and no EGFR, MET, HER2 mutation or amplification, it has

been classified later as a mesenchyme-like phenotype, although it expresses significant levels of E-cadherin.

Section 3.2 Protein expression of PRMT5 in mesenchyme-like and bronchial epithelial phenotype cell lines, by western blotting and immunocytochemistry

To compare the protein expressions of PRMT5 between bronchial epithelial phenotype and mesenchyme-like phenotype cell lines, and to confirm the RNA expression data, I performed western blot analysis using 6 lung adenocarcinoma cell lines, containing three mesenchyme-like phenotype; H522, H1651 and A549, and three bronchial epithelial phenotype with EGFR mutations; HCC4006, H1650 and PC3 (Fig 2). I tested the protein expression levels of PRMT5, vimentin, E-cadherin, TTF-1, CK7, and MUC1 of the 6 cell lines. PRMT5 protein expression was more in the mesenchyme-like cell lines which frequently showed high expression of vimentin and low expressions of bronchial epithelial markers (TTF-1, CK7, MUC1 and E-cadherin).

Next, I performed immunocytochemical analysis, using these 6 cell lines. Interestingly I found that PRMT5 expression was predominant in the cytoplasm in mesenchyme-like phenotype cell lines, whereas it was predominantly expressed in the nucleus but faint in the cytoplasm in bronchial epithelial phenotype with EGFR mutations (HCC4006, H1650, and PC3) (Fig.3). This result suggested that the cytoplasmic expression of PRMT5 may be associated with EMT and/or wild type EGFR.

Section 3.3 Immunohistochemical expression of PRMT5 in primary lung adenocarcinoma tissues

I used tissue microarray sections of primary lung adenocarcinoma cases (n=130) to examine immunohistochemical expression patterns of PRMT5, and their relationship with (i) histopathological subtypes, (ii) genetic status of EGFR (iii) expressions of bronchial epithelial markers (TTF-1, CK7, MUC1 and E-cadherin), and (iv) clinic-pathological factors. Using the criterion described in Materials and Methods, among 130 cases, 43 showed high cytoplasmic expression and low nuclear expression of PRMT5, 30 showed low cytoplasmic expression and high nuclear expression of PRMT5, and 53 showed low cytoplasmic expression and low nuclear expression of PRMT5. Though 4 cases showed both cytoplasmic and nuclear high expression levels of PRMT5, there was significantly inverse correlation between cytoplasmic and nuclear expressions of PRMT5 in 130 cases (p=0.0002 Chi-square test) . Then we examined the expression levels of PRMT5 in cytoplasm and nucleus in various histopathological subtypes. Normal alveolar epithelia were negative for PRMT5, whereas the nuclear expression of PRMT5 was high in the well differentiated adenocarcinoma component, that is, lepidic growth component (6 of 17, 35%), which less frequently showed the high cytoplasmic expression of PRMT5 (1 of 17, 6%) (Fig. 4A, Table 3). Moderately differentiated adenocarcinoma components, that is, acinar or papillary adenocarcinomas, showed high nuclear expression of PRMT5 in 25 of 81 cases (31%), and high cytoplasmic expression of PRMT5 in 21 of 81 cases (26%) (Fig. 4B, 4C, and Table 3), this indicates no cytoplasmic or nuclear predominance of expression as the tumor moves from well to poorly differentiated state. Poorly differentiated adenocarcinoma component, that is, solid adenocarcinoma with mucin, frequently showed high cytoplasmic expression of PRMT5 (23 of 29, 79%) and less frequently showed high nuclear

expression of PRMT5 (3 of 29, 10%) (Fig. 4D, and Table 3), as well the cytoplasmic predominance was seen in invasive mucinous adenocarcinoma (2 of 3, 66%) (Fig. 4E, and Table 3). PRMT5 sometimes showed heterogeneous staining pattern, typically showing nuclear positive staining in well to moderately differentiated adenocarcinoma components, while showing cytoplasmic staining in poorly differentiated adenocarcinoma components (Fig. 4F).

Furthermore I checked the correlations between histological grades (low-grade, intermediate-grade, and high-grade, described in Materials and Methods) and PRMT5 expression levels in cytoplasm and nucleus, respectively. The cases with high cytoplasmic PRMT5 expression significantly showed higher-grade tumor than those with low cytoplasmic PRMT5 expression ($p<0.0001$), whereas the cases with high nuclear PRMT5 expression significantly showed lower-grade tumor than those with low nuclear PRMT5 expression ($p=0.0444$) (Table 4).

Then to check the significance of PRMT5 cytoplasmic expression, we classified 130 cases into three groups according to PRMT5 expression pattern (Table 5); C+ group, comprises the cases showing high cytoplasmic expression levels with or without nuclear expressions (n=47), C-N+ group, comprising cases showing low cytoplasmic and high nuclear expression levels (n=30), and C-N- group, comprising cases showing low cytoplasmic and low nuclear expression levels (n=53). We compared histological grades and this group classification, then we found that C+ group significantly showed higher grades than C-N+ and C-N- groups ($p<0.0001$), however, there was no significant difference between C-N+ group and C-N- group ($p=0.3780$), as shown in Table 5. These results suggested that the nuclear localization of PRMT5 may not be correlated with the maintenance of a differentiated phenotype, whereas the cytoplasmic localization of PRMT5 appears to be significant in the processes of dedifferentiation.

Next, we tested the correlation of cytoplasmic expression of PRMT5 with EGFR status and bronchial epithelial markers (TTF-1, CK7, MUC1, and E-cadherin) (Table 6). We found that high cytoplasmic PRMT5 expression was significantly correlated with low expression levels of TTF-1, CK7, MUC1 (membranous), and E-cadherin, and high expression level of depolarized MUC1 (Fig.5A-E, Table 6). Usually with dedifferentiation, lung adenocarcinoma loose the membranous MUC1, but in some cases they also show expression of depolarized MUC1 in cytoplasm, and this was seen in Fig. 5E . The cases with high cytoplasmic expression levels of PRMT5, not significantly, but tended to show wild type EGFR (p=0.0880) (Table 6). Fig.5F-J shows typical immunohistochemical expression patterns of TTF-1, CK7, E-cadherin, and MUC1 in lepidic growth components with high nuclear expression of PRMT5 and high expression of bronchial epithelial markers.

Then we checked the effect of the cytoplasmic expression levels of PRMT5 on clinicopathological factors, it is found to be significantly correlated with vessel invasion (Table 7).

Section 3.4 PRMT5 expression and patient survival

In order to check the significance of cytoplasmic PRMT5 expression on patients' survival, we performed a survival analysis using the Kaplan-Meier method. The patients with high cytoplasmic PRMT5 expressions had significantly poorer survival rates compared to those with low cytoplasmic PRMT5 expressions (Fig. 6). Furthermore we compared the survival of the cases of the above-mentioned three groups; Group A, Group B and Group C. We found that Group A (the cases with high cytoplasmic PRMT5 expressions) had the worst survival compared

to the others, and among the cases with low cytoplasmic PRMT5 expressions, Group B (the cases with high nuclear PRMT5 expressions) showed poorer prognosis than Group C (the cases with low nuclear PRMT5 expressions) (Fig. 7).

It is difficult to determine whether cytoplasmic PRMT5 expression is independently correlated with survival using multivariate analysis because the correlation between cytoplasmic PRMT5 expression and histological grading was too strong according to a statistician point of view. We speculated that the cytoplasmic expression of PRMT5 may contribute to a poor prognosis by promoting high-grade transformation and EMT.

Chapter 4. Discussion

To my knowledge this is the first report demonstrating the correlation between cytoplasmic expression of PRMT5 and EMT in lung adenocarcinoma. There have been a few reports describing the role of cytoplasmic PRMT5 in cancer. One of them reported the accumulation of PRMT5 with androgen receptor (AR)-interacting protein p44 in the cytoplasm of prostatic cancer cells and tissues. The authors showed that both proteins are required for the growth of prostatic cancer cells, furthermore they performed subcellular localization assays designed to span the entire open-reading frame of the PRMT5 protein, it revealed the presence of three nuclear exclusion signals (NESs) in the PRMT5 protein [(28)].Another report also stated that cytoplasmic p44 and PRMT5 are associated with seminoma and Leydig cell tumor development [(32)]. A recent paper identified the accumulation of PRMT5 in the cytoplasm in metastatic melanoma cells, they relate the cytoplasmic expression to the interaction with Mep50 (Methylosome protein 50) [(33)]. However no study focused on the role of PRMT5 in EMT in lung adenocarcinoma.

PRMT5 known to be located in the nucleus and cytoplasm, it has different substrates in each compartment and this affects its function. In my study I found that high cytoplasmic expression is closely related to high-grade subtypes of primary lung adenocarcinomas, and highly expressed in the mesenchyme-like phenotype cell lines which express high vimentin, and also correlated to loss of E-cadherin and the other bronchial epithelial markers (TTF-1, CK7, and MUC1), which suggests it's relation to EMT.

In my study I focused on the role of methyltransferases and demethylases especially PRMT5 in lung adenocarcinomas which show EMT features. I found that cytoplasmic

PRMT5 may be involved in tumorigenesis of such type of cancers. So the question would be, how cytoplasmic PRMT5 can affect lung adenocarcinomas with EMT features? To answer this question a link between the role of cytoplasmic PRMT5 and EMT should be identified. In the following paragraphs I will try to explain the possibilities from the previous reports.

PRMT5 is known to be expressed in the cytoplasm in ES cells (embryonic stem cells) to maintain pluripotency and stemness through methylation of cytosolic histone H2A during mouse development [(34)]. On the other hand cells with EMT features have been identified to acquire stem cells properties [(35)], so This finding justifies the accumulation of PRMT5 in the cytoplasm during EMT. Histone H2A has many variants, including H2A.Z, H2A.X, macroH2A and H2A.Bbd. Histone H2A and its variants are actively exchanged, and the equilibrium between their removal and incorporation determines their abundance in chromatin during development. Histone H2A is involved in transcriptional activation, acts as an anti-silencing factor at heterochromatin boundaries, involved in epigenetic memory, DNA repair process, and X chromosome inactivation

The EMT process is characterized by (i) changes of differentiation markers from cell-cell junction proteins and cytokeratin intermediate filaments to vimentin filaments and fibronectin, i.e. reorganization of the cell cytoskeleton [(9)] and we could closely relate the vimentin expression to the increase in PRMT5 expression in mesenchyme-like cell lines. It is known that protein methylation by methyltransferases can affect substrate protein expression, stability or interactions with other proteins. So PRMT5 might affect the vimentin expression through methylation in the cytoplasm. (ii) activation of EMT-inducing transcription factors [(9)]. In the cytoplasm, PRMT5 is known to methylate some of the sm proteins and this methylation facilitates the binding of the survival motor neuron protein (SMN) which is important for snRNP core particle assembly, the snRNPs form the core of the splicesome which is responsible for the

pre-mRNA splicing [36]. Thus PRMT5 is an essential regulator of splicing. It is known that alternative splicing affects tumor apoptosis, invasion and metastasis [37], so it is rationale to think that PRMT5 might affect either the function or expression of some EMT-related genes through alternative splicing leading to EMT features and cancer invasion.

From the entire above mechanisms I can realize that cytoplasmic PRMT5 could be related to EMT, however in this study I need to find the cytoplasmic interacting proteins to figure out the exact mechanism by which PRMT5 perform its function in lung adenocarcinoma.

I also found that EGFR-mutated tumors, not significantly, but tended to show low cytoplasmic PRMT5 expressions. Cytoplasmic PRMT5 was reported to methylate EGFR Arg 1175, and abolishment of this methylation enhanced ERK activation [38], which suggested that cytoplasmic PRMT5 may be, to some extent, incompatible for the survival of EGFR mutated tumors because of its inhibitory effect on the activation of ERK through the methylation of EGFR Arg 1175 [38].

Furthermore I showed that cytoplasmic accumulation of PRMT5 is associated with poor survival of patients.

In the present study, I showed that nuclear expression of PRMT5 was more frequent in low-grade tumor and less frequent in high-grade tumor. However, among the cytoplasmic PRMT5 negative cases, nuclear PRMT5 positive cases showed poorer prognosis than nuclear PRMT5 negative cases. These results suggest that nuclear accumulation of PRMT5 may play an important role in the early steps of malignant progression, and its localization may be changed from the nucleus to the cytoplasm during dedifferentiation and EMT (Fig 8).

Taking all these results into account, I believe that the cytoplasmic expression of PRMT5 in lung adenocarcinoma is of great importance in the processes of EMT and loss of bronchial epithelial phenotype of lung adenocarcinoma. Furthermore; epigenetic therapy aimed at inhibiting PRMT5 may be an effective therapy to treat tumors with EMT features. However the mechanism needs further studies.

The mechanism of EMT and cancer invasion is complex and probably not a single molecule would be responsible, but knowing additional information about the molecular mechanism is important to support identifying the full view of cancer invasion and metastasis in lung adenocarcinoma. If we did so, and all molecules involved in the different steps of EMT are identified I think a combination therapy will be of great help.

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Table 1: 2011 International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society international multidisciplinary classification of lung adenocarcinoma

1- Pre-invasive lesions
- Atypical adenomatous hyperplasia
- Adenocarcinoma *in situ* (≤3 cm, formerly bronchioloalveolar carcinoma)
- Non-mucinous and/or mucinous
2- Minimally invasive adenocarcinoma (a ≤3 cm lepidic predominant tumor with ≤5 mm invasion)
- Non-mucinous and/or mucinous
3- Invasive adenocarcinoma
- Lepidic predominant (formerly non-mucinous bronchioloalveolar carcinoma pattern)
- Acinar predominant
- Papillary predominant
- Micropapillary predominant
- Solid predominant with mucin
4- Variants
- Invasive mucinous adenocarcinoma (formerly mucinous bronchioloalveolar carcinoma) and Mixed mucinous/non-mucinous
- Colloid
- Fetal
- Enteric

Table 2: Histone methyltranseferase and demethylase genes, which met the following requirement: (correlation coefficient with vimentin – correlation coefficient with E-cadherin) x ½> 0.3 or <-0.3

Gene symbol	Probe number	Correlation coefficient*
PRMT5	1564520_s_at	0.30985
PRMT5	1564521_x_at	0.35714
PRMT5	217786_at	0.30459
SETDB2	235338_s_at	0.30372
SUV39H2	1554572_a_at	0.31779
JMJD1A	212689_s_at	0.39253
PRMT6	223275_at	-0.37497

Abbreviations: PRMT5 Protein arginine methyltransferase 5, SETDB2: SET domain bifurcated 2, SUV39H2: Suppressor of variegation 3-9 homolog 2, JMJD1A: Jumonji domain containing 1A, PRMT6: Protein arginine methyltransferase 6.

*Correlation coefficient > or < 0.03 is significant.

Table 3: Correlations between expression levels of PRMT5 in cytoplasm and nucleus and histopathological subtypes of primary lung adenocarcinomas

Subtypes		C +	C-
Lepidic growth component*	N +	0	6
	N -	1	10
Acinar adenocarcinoma component¶	N +	0	4
	N -	9	12
Papillary adenocarcinoma component¶	N +	1	20
	N -	11	24
Solid adenocarcinoma component±	N +	3	0
	N -	20	6
Invasive mucinous adenocarcinoma component±	N +	0	0
	N -	2	1
	Total	47	83

* Lepidic component: low grade tumor, ¶ Acinar and Papillary components: intermediate grade tumors, ±Solid and invasive mucinous: high grade tumors.

Abbreviations, C+: high cytoplasmic expression of PRMT5, C-: low cytoplasmic expression of PRMT5, N+: high nuclear expression of PRMT5, and N -: low nuclear expression of PRMT5.

Table 4: Correlations between expression levels of PRMT5 in cytoplasm and nucleus and histopathological grades of primary lung adenocarcinomas

Histological grades	C +	C -	*P-value*	N +	N -	*P-value*
Low-grade	1	16	<0.0001*	6	11	0.0444*
Intermediate-grade	21	60		25	56	
High-grade	25	7		3	29	

Abbreviations, C +: high cytoplasmic expression of PRMT5, C -: low cytoplasmic expression of PRMT5, N +: high nuclear expression of PRMT5, and N -: low nuclear expressions of PRMT5.
*P-value < 0.0167 is significant (Mann-Whitney U-test).

Table 5: Correlation between the grades of lung adenocarcinoma and groups which defined by PRMT5 expression pattern

Histological grades	C+ group	C-N+ group	C-N- group	*P-value*
Low-grade	1	6	10	C+ *vs.* C-N+: < 0.0001*
Intermediate-grade	21	24	36	C-N+ *vs.* C-N-: 0.3780
High-grade	25	0	7	C+ *vs.* C-N-: < 0.0001*

Abbreviations, C+ group: cases showing high cytoplasmic expression levels with or without nuclear expressions (n=47), C-N+ group: cases showing low cytoplasmic and high nuclear expression levels (n=30), and C-N- group, that means the cases showing low cytoplasmic and low nuclear expression levels (n=53). *P-value < 0.0167 is significant (Mann-Whitney U-test).

Table 6: Correlations between expression levels of cytoplasmic PRMT5 and EGFR mutations and the expressions of bronchial epithelial markers

	Cytoplasmic PRMT5		
	High	Low	*P*-value
EGFR mutations			
- positive	12	31	0.0880
- negative	25	31	
E-cadherin			
- high level	42	80	0.0377*
- low level	4	1	
TTF-1			
- high level	30	75	<0.0001*
- low level	17	4	
CK7			
- high level	34	74	0.0140*
- low level	14	9	
MUC1 (membranous)			
- high level	21	62	0.0006*
- low level	26	21	
MUC1 (depolarized)			
- high level	12	3	0.0002*
- low level	35	80	

Abbreviations, EGFR: Epidermal growth factor receptor, TTF-1: Thyroid transcription factor 1, CK7: Cytokeratin 7, MUC1 Mucin 1. *P-value < 0.05 is significant (Chi-square test).

Table 7: Correlation between expression levels of cytoplasmic PRMT5 and clinicopathological factors.

	Cytoplasmic PRMT5		
	High	Low	*P*-value
Pathological stage			
- Stage IA	14	38	0.0887
- Stage IB-IV	32	45	
T-stage			
- T1	16	42	0.0680
- T2,T3,T4	31	41	
Nodal involvement			
- positive	12	21	0.8858
- negative	34	56	
Lymphatic invasion			
- positive	12	17	0.5064
- negative	35	66	
Vessel invasion			
- positive	24	27	0.0376*
- negative	23	56	
Pleural invasion			
- positive	26	35	0.1489
- negative	21	48	
Dissemination			
- positive	0	2	0.2835
- negative	47	81	
Tumor size			
- ≤ 3cm	16	22	0.3640
- > 3cm	31	61	
Pulmonary metastasis			
- positive	4	6	0.5916
- negative	43	78	
Smoking Index			
- ≤ 600	21	34	0.7410
- > 600	24	44	

*P-value < 0.05 is significant (Chi-square test).

Figure 1: Gene expression of PRMT5 in cell lines

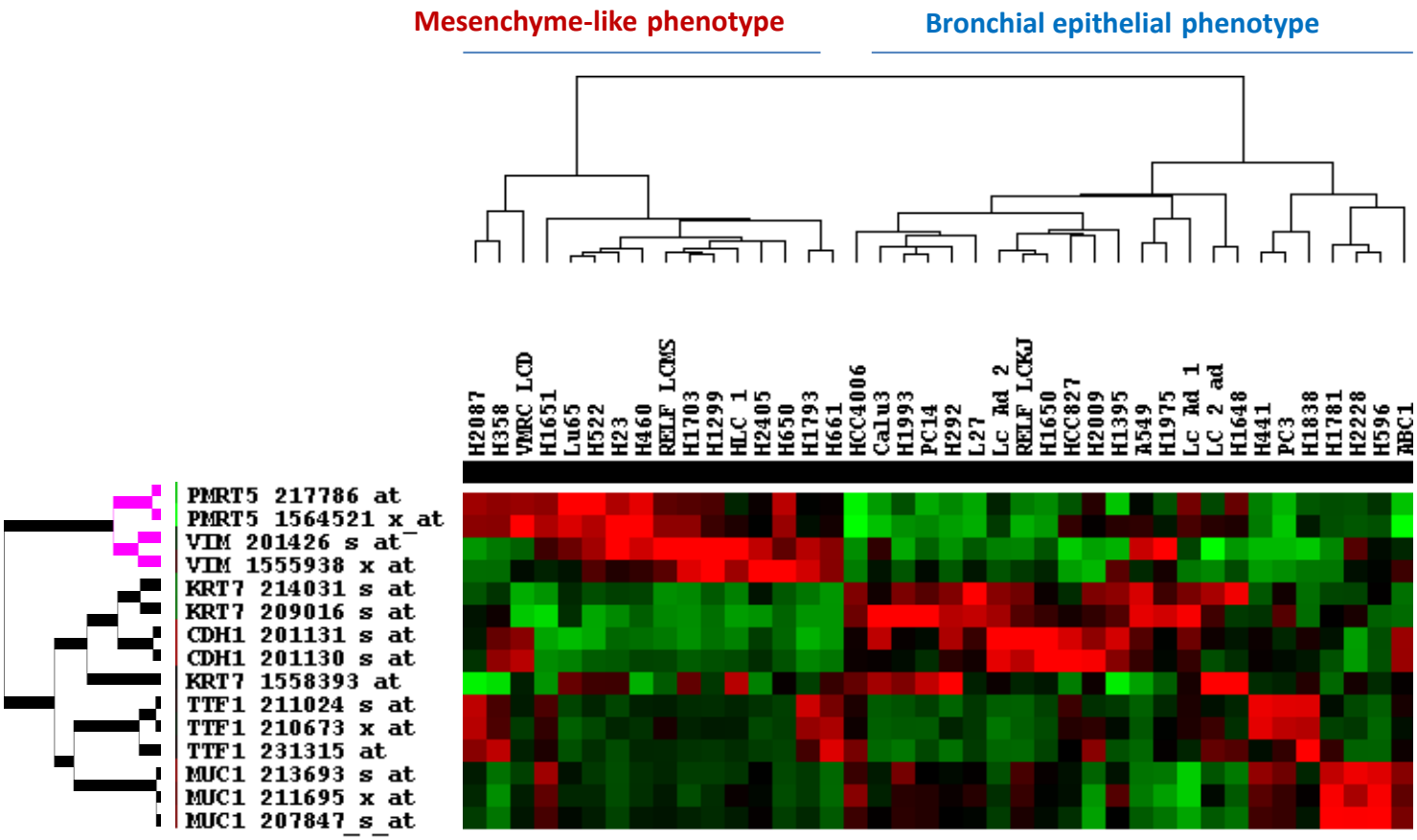


Figure 1:

Shows cluster analysis of 40 cell lines. Cell lines are clustered into 2 groups: mesenchyme-like cell lines (right cluster) and bronchial epithelial phenotype cell lines (left cluster).PRMT5, VIM (Vimentin), KRT7 (CK7 or Cytokeratin 7), CDH1 (E-Cadherin), TTF-1 (Thyroid transcription factor 1), MUC1 (Mucin1) expression is shown.PRMT5 expression is related to vimentin and inversely related to other epithelial markers (KRT7, CDH1, TTF-1, MUC1).

Figure 2: Protein expression of PRMT5 in cell lines

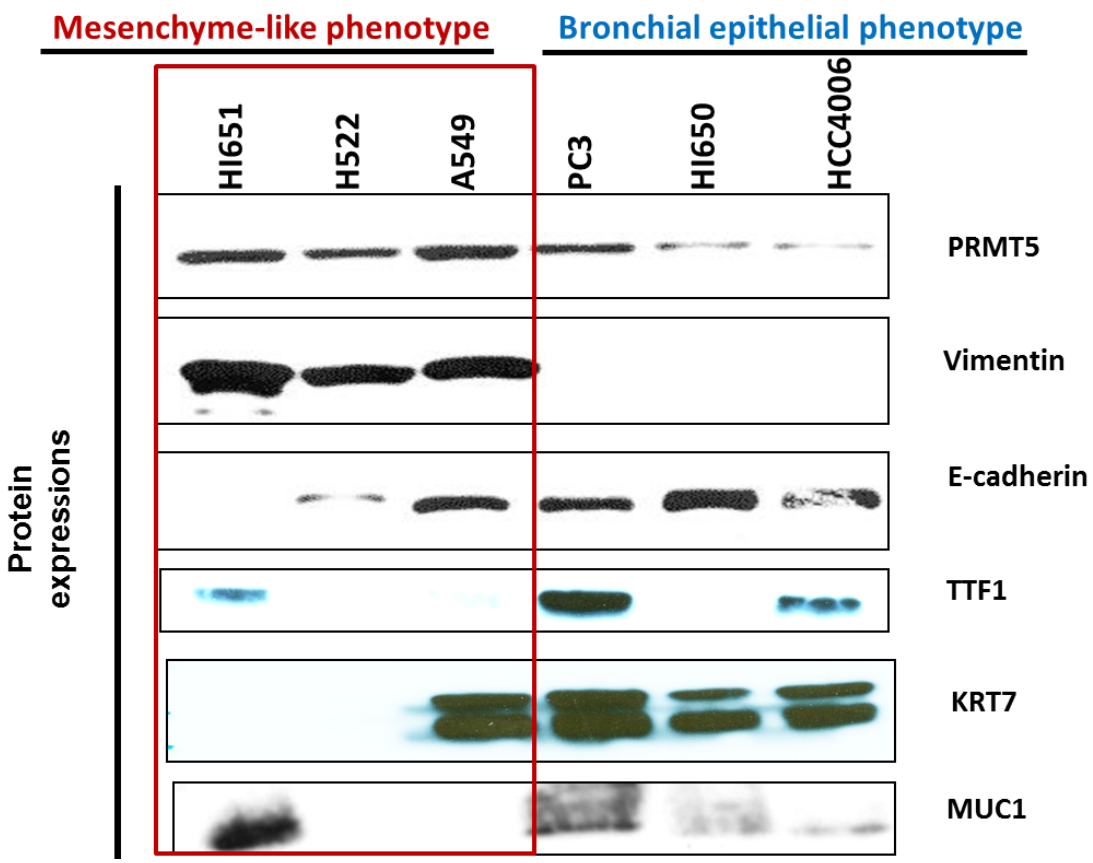


Figure 2:

Shows Western blot in 6 cell lines. 3 are mesenchyme-like phenotype cell lines (H1651, H522, A549), and the other 3 are bronchial epithelial phenotype cell lines (PC3, H1650, HCC4006).It shows the protein expression of PRMT5, vimentin, E-Cadherin, KRT7 (CK7, cytokeratin 7), and TTF-1 (Thyroid transcription factor-1) in both types of cell lines.

Figure 3: Immunocytochemical expression of PRMT5

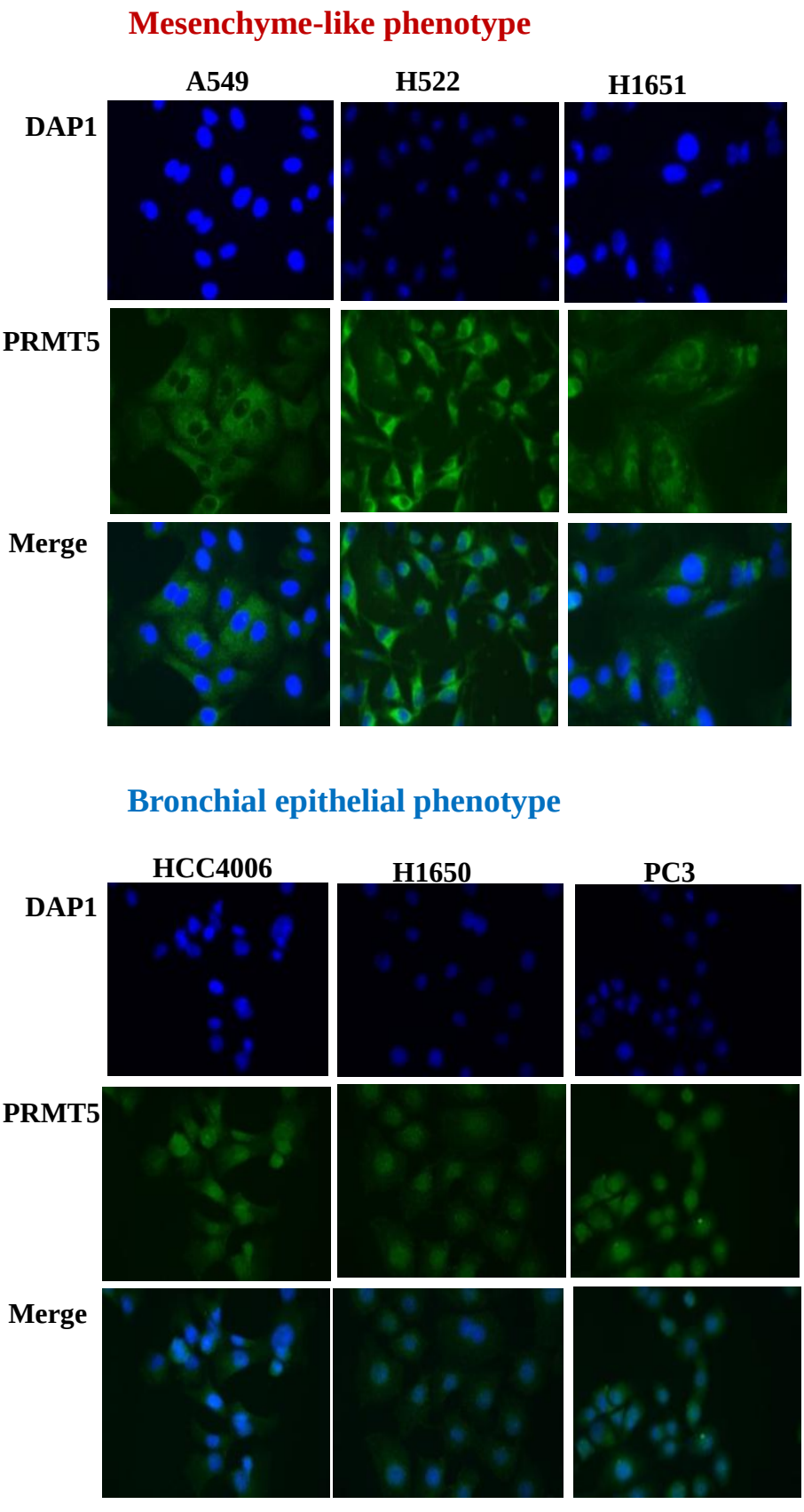


Figure 3:

Immunocytochemical expression of PRMT5 in 6 cell lines. Immunofluorescent detection of PRMT5 is shown in green. Nuclear staining (DAPI) is shown in blue. Upper panel: shows the expression pattern of PRMT5 in mesenchymal-like phenotype cell lines (A549, H522, and H1651). PRMT5 expression is predominantly cytoplasmic. Lower panel: shows the expression pattern of PRMT5 in bronchial epithelial phenotype cell lines (PC3, H1650, and HCC4006). PRMT5 expression is predominantly in nucleus. (Magnifications x400)

Figure 4: PRMT5 expression in primary lung adenocarcinoma tissues

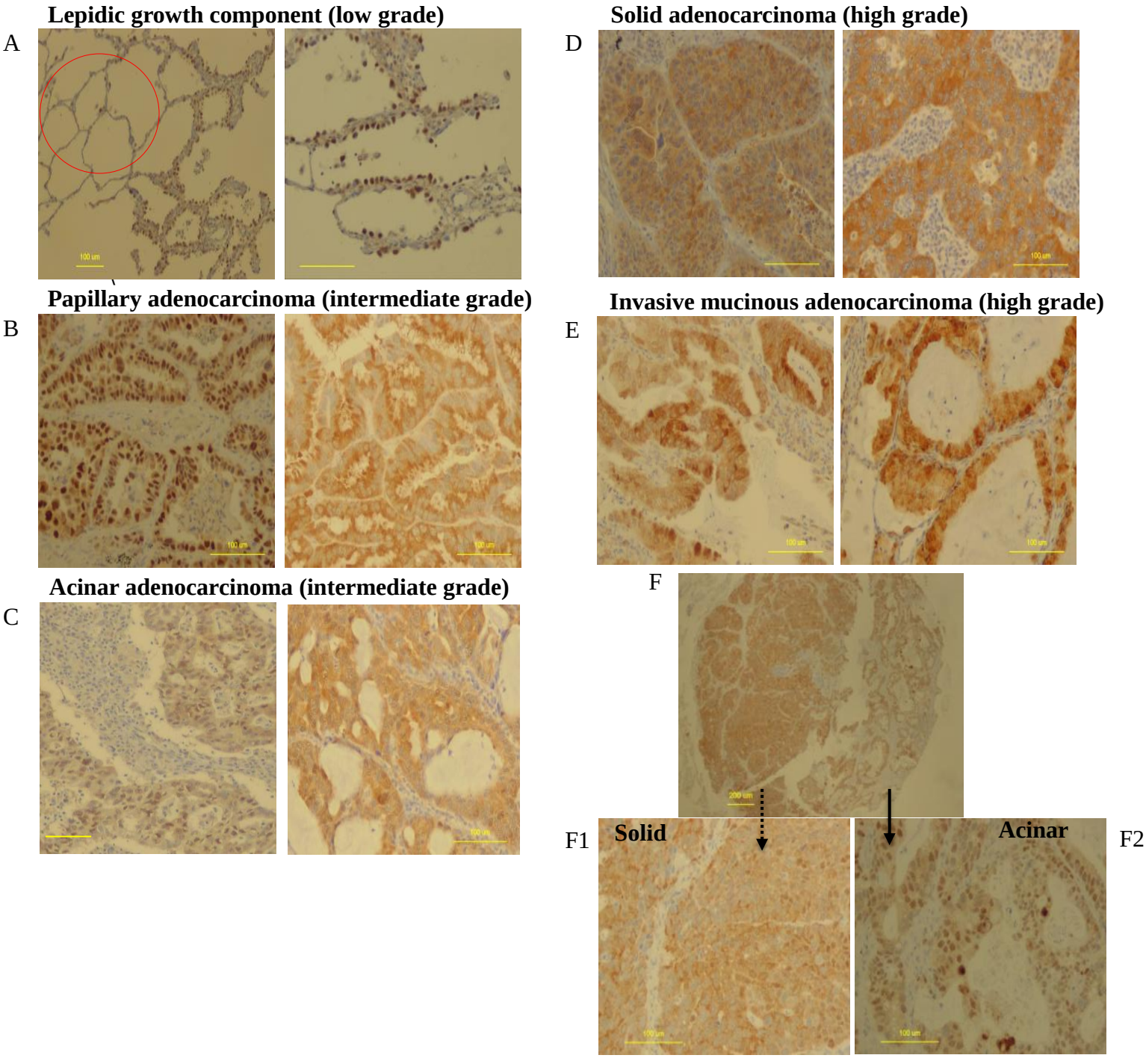


Figure 4:

Fig. 4A: Left panel: circle shows normal lung epithelium which shows no PRMT5 expression. Well differentiated adenocarcinoma component (lepidic growth component) is positive for PRMT5 (right side) and shows nuclear expression of PRMT5 (magnification x100).Right panel shows a magnified view for the lepidic growth component, which shows nuclear expression of PRMT5. (Magnification x200)

Fig. 4B: Intermediate grade tumor (papillary adenocarcinoma).Left panel: nuclear expression of PRMT5 is observed. Left panel: cytoplasmic expression of PRMT5 is observed. (Magnification x200). No nuclear or cytoplasmic predominance is observed.

Fig. 4C: Intermediate grade tumor (Acinar adenocarcinoma).Left panel: nuclear expression of PRMT5 is observed. Right panel: cytoplasmic expression of PRMT5 is observed. (Magnification x200).

Fig. 4D: High grade tumor (solid adenocarcinoma).Both left panel and right panels show cytoplasmic expression of PRMT5. (Magnification x200).

Fig 4E: Invasive mucinous adenocarcinoma also frequently shows cytoplasmic expression of PRTM5. (Magnification x200)

Fig. 4F: Heterogeneous PRMT5 expression in lung adenocarcinoma. Fig. 4F shows a low-power field of invasive adenocarcinoma with mixed subtypes. Left side shows solid adenocarcinoma components (dashed black arrow), and right side shows acinar adenocarcinoma and lepidic growth components (black arrow). (Magnification x40) Fig. 4F1 shows a high-power field of solid adenocarcinoma components, which show cytoplasmic expression of PRMT5.

(Magnification x200) Fig. 4F2 shows a high-power field of acinar adenocarcinoma and lepidic growth components, which show nuclear expression of PRMT5. (Magnification x200)

Figure 5: Immunohistochemical expression of PRMT5, E-Cadherin, TTF-1, CK7, and MUC1in the same tissue samples.

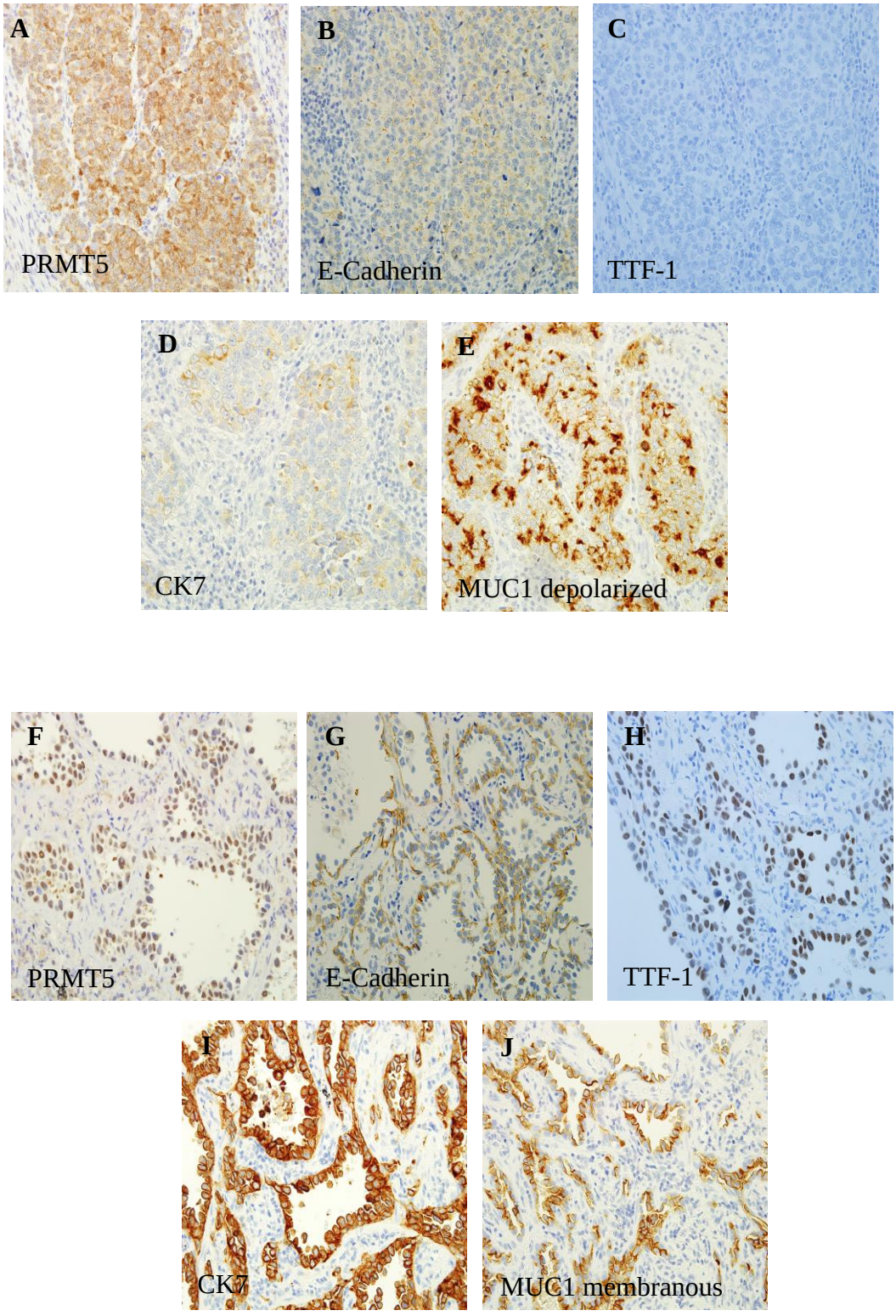


Figure 5:

Fig. 5A-E: Case of solid adenocarcinoma with mucin (high-grade subtype).

Fig. 5A: shows cytoplasmic expression of PRMT5.

Fig. 5B: shows low level expression of E-cadherin in membrane.

Fig. 5C: shows low level expression of TTF-1 in nucleus.

Fig. 5D: shows low level expression of CK7 in membrane and cytoplasm.

Fig. 5E: shows low level expression of membranous MUC1, and shows depolarized (cytoplasmic) expression pattern of MUC1.

Fig. 5F-J: Case of adenocarcinoma in situ (low-grade subtype).

Fig. 5F: shows nuclear expression of PRMT5.

Fig. 5G: shows high level expression of E-cadherin in membrane.

Fig. 5H: shows high level expression of TTF-1 in nucleus.

Fig. 5I: shows high level expression of CK7 in membrane and cytoplasm.

Fig. 5J: shows high level expression of MUC1 in apical membrane.

All figures magnifications is x200

Figure 6: Overall survival curve according to cytoplasmic PRMT5 expression

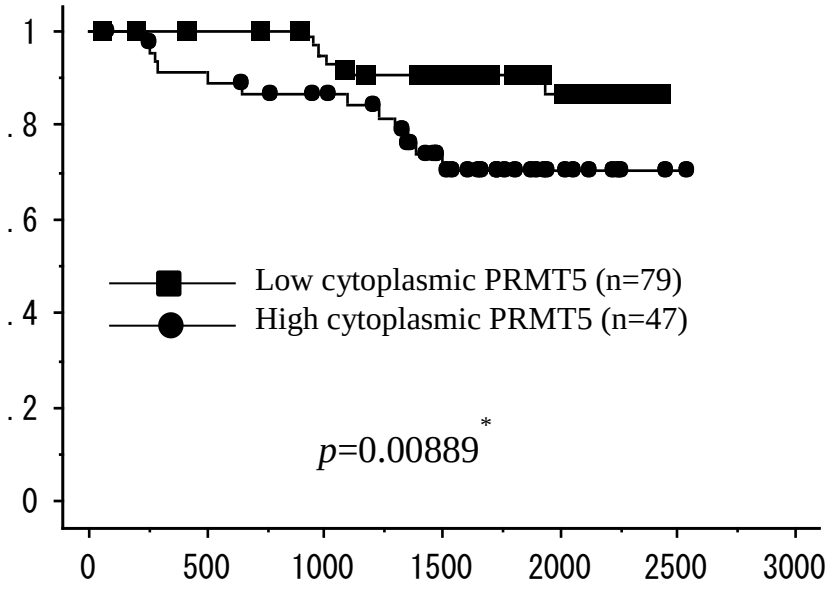


Fig. 6:

Survival analysis for low cytoplasmic PRMT5 cases (n: number of cases=79), and high cytoplasmic PRMT5 cases (n: number of cases=47). $^{*}P$-value <0.05 is significant.

Figure 7: Overall survival curve according to groups with different PRMT5 expressions

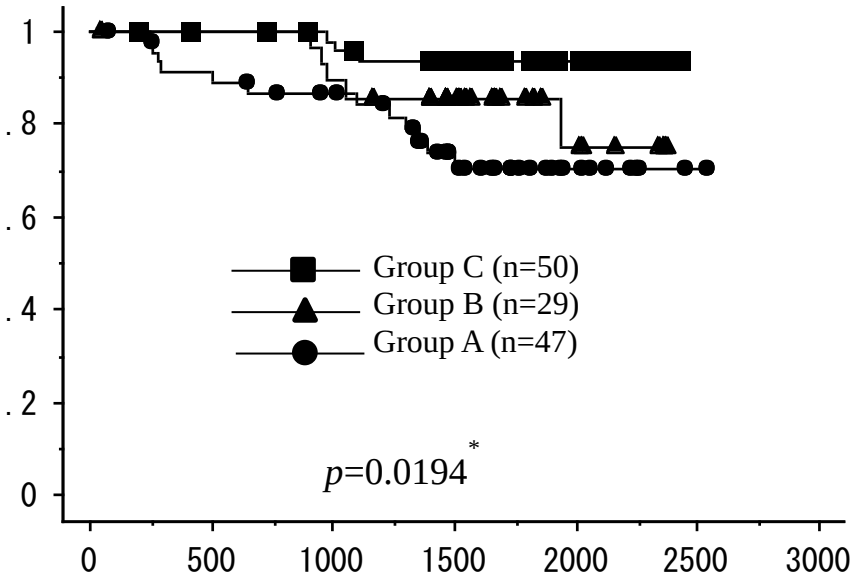
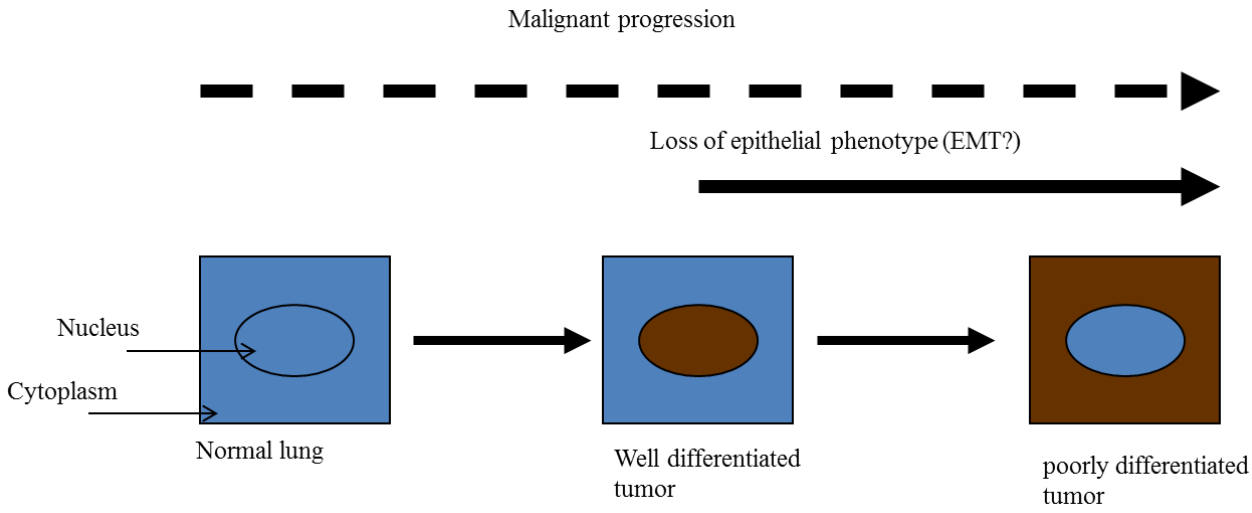


Fig. 7:

Survival analysis among 3 groups of cases. Group A: cases which show both positive or negative nuclear expression of PRMT5 and positive cytoplasmic expression. Group B: cases which show positive nuclear expression of PRMT5 and negative cytoplasmic expression. Group C: cases which show negative PRMT5 expression in both nucleus and cytoplasm. $^{*}P$-value <0.05 is significant.

Figure 8 : Hypothetical scheme of the role of PRMT5



Brown color: PRMT5 expression

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