การตรวจหาสภาวะเหนือพันธุกรรมของยีน SHP-1 promoter 2 โดยการเติมหมู่เมททิลในพลาสม่าของผู้ป่วยมะเร็งปอดและบทบาททางคลินิก

นางสาว ชนิดา วินะยานุวัติคุณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DETECTION OF ABERRANT SHP-1 PROMOTER 2 METHYLATION, AN IMPLICATION IN ADVANCED NON-SMALL CELL LUNG CANCER

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ชนิดา วินะยานุวัติคุณ : การตรวจหาสภาวะเหนือพันธุกรรมของยีน SHP-1 promoter 2 โดยการเติมหมู่เมททิลในพลาสม่าของผู้ป่วยมะเร็งปอดและบทบาททางคลินิก. (DETECTION OF ABERRANT SHP-1 PROMOTER 2 METHYLATION, AN IMPLICATION IN ADVANCED NON-SMALL CELL LUNG CANCER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.นพ. ดร. วิโรจน์ ศรีอุฬารพงศ์ , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.นพ.ดร. อภิวัฒน์ มุทิรางกูร, 105 หน้า.

หลักการและเหตุผล สารพันธุกรรมที่ตรวจพบในพลาสม่าของคนปกติพบว่าส่วนประกอบหลักนั้นเป็นสารพันธุกรรมจากเม็ดเลือดขาว ดังนั้นการตรวจหาสภาวะเหนือพันธุกรรมของ SHP-1 promoter-2 (SHP1P2) ที่มีการเติมหมู่เมททิลซึ่งมีความจำเพาะกับเซลมะเร็งเซลเยื่อบุผิวแต่ไม่พบสภาวะเหนือพันธุกรรมดังกล่าวในเม็ดเลือดขาวจะสามารถบ่งบอกถึงสารพันธุกรรมที่มีแหล่งที่มาจากเซลมะเร็งเยื่อบุผิวในพลาสม่าของผู้ป่วยมะเร็งเยื่อบุผิว ซึ่งอาจจะนำไปสู่การใช้การเป็นตัวบ่งชี้ทางชีวโมเลกุลในการติดตามการตอบสนองการรักษาหรือช่วยในการวินิจฉัย นอกจากนี้ยังอาจใช้ศึกษาความสำคัญของปริมาณสารพันธุกรรมจากเซลมะเร็งและเซลเม็ดเลือดขาวในพลาสม่าของผู้ป่วยมะเร็งเยื่อบุผิวซึ่งในที่นี้ศึกษาโดยใช้มะเร็งปอดชนิดเซลไม่เล็กเป็นต้นแบบ

วิธีการศึกษาวิจัย ผู้วิจัยได้ทำการวัดปริมาณสภาวะเหนือพันธุกรรมของยีน SHP1P2 ที่มีการเติมหมู่เมททิลโดยวิธีการ real-time polymerase chain reaction ร่วมกับ dual hybridization probeในพลาสม่าของผู้ป่วยมะเร็งปอดชนิดเซลไม่เล็กระยะลุกลามจำนวน 58 คนและคนปกติ 52 คนโดยได้ทำการวิเคราะห์ควบคู่ไปกับข้อมูลทางคลินิก

ผลการศึกษาวิจัย ระดับสภาวะเหนือพันธุกรรมของยีน SHP1P2 ที่มีการเติมหมู่เมททิลในพลาสม่าของผู้ป่วยมะเร็งปอดชนิดเซลไม่เล็กนั้นมีปริมาณสูงอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับคนปกติซึ่งแทบไม่พบสภาวะเหนือพันธุกรรมที่มีการเติมหมู่เมททิลของยีนดังกล่าวเลย พบว่าค่ามัธยฐาน 0.77 ng ml^{-1} [0–26.5 ng ml^{-1}] ในผู้ป่วยมะเร็งปอดชนิดเซลไม่เล็ก นอกจากนี้สภาวะเหนือพันธุกรรมของยีน SHP1P2 ที่มีการเติมหมู่เมททิลยังสามารถช่วยในการพยากรณ์โรค กล่าวคือผู้ป่วยที่มีระดับน้อยกว่า 0.7 ng ml^{-1}พบว่ามีพยากรณ์โรคที่ดีกว่ากลุ่มที่มีค่าดังกล่าวมากกว่า 0.7 ng ml^{-1} โดยค่ามัธยฐานของระยะเวลาที่อยู่รอด (12.6 vs. 7.6 เดือน, p = 0.01) และค่ามัธยฐานของระยะเวลาที่โรคลุกลาม (5.2 vs. 2.6 เดือน, p = 0.009) การวิเคราะห์หลายตัวแปรโดยรวมข้อมูลทางคลินิกพบว่าระดับของสภาวะเหนือพันธุกรรมของยีน SHP-1 promoter 2 ที่มีเติมหมู่เมททิลเป็นปัจจัยเพียงอย่างเดียวมีความสำคัญอย่างมีนัยทางสถิติต่อการพยากรณ์โรค นอกเหนือจากนี้ระดับสารพันธุกรรมทั้งหมดในพลาสม่ายังสามารถใช้พยากรณ์โรคหากแต่มีความจำเพาะน้อยกว่าสภาวะเหนือพันธุกรรมของยีน SHP-1 promoter 2 ที่มีการเติมหมู่เมททิล, การเปลี่ยนแปลงของสารพันธุกรรมทั้งหมดในพลาสม่าสัมพันธ์ไปกับผลการตอบสนองของการรักษาด้วยยาเคมีบำบัด โดยอาจเป็นผลจากปริมาณสารพันธุกรรมของเซลมะเร็งเยื่อบุผิวซึ่งสัมพันธ์กับปริมาณเซลมะเร็ง และเซลเม็ดเลือดขาวซึ่งอาจเป็นปฏิสัมพันธ์ระหว่างเซลมะเร็งกับการอักเสบในร่างกาย

สรุป การวัดค่าสภาวะเหนือพันธุกรรมของยีน SHP1P2 ที่มีการเติมหมู่เมททิลในพลาสม่าของผู้ป่วยมะเร็งปอดชนิดเซลไม่เล็กมีแนวโน้มที่จะเป็นตัวบ่งชี้ทางชีวโมเลกุลเพื่อการวินิจฉัยและการทำนายพยากรณ์โรค เพิ่มเติมจากระดับสารพันธุกรรมใน พลาสม่า การจำแนกผู้ป่วยโดยการใช้ตัวบ่งชี้ทางชีวโมเลกุลเพิ่มเติมอาจมีส่วนช่วยในการวางแผนการรักษาผู้ป่วยต่อไป

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5187771020 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS : SHP-1 PROMOTER 2 METHYLATION / PLASMA BIOMARKER / NON-SMALL CELL LUNG CANCER / EPITHELIAL SPECIFIC METHYLATION MARKER / BIOLOGY OF CIRCULATING DNA

CHANIDA VINAYANUWATTIKUN : DETECTION OF ABERRANT SHP-1 PROMOTER 2 METHYLATION, AN IMPLICATION IN ADVANCED NON-SMALL CELL LUNG CANCER. ADVISOR : ASST. PROF. VIROTE SRIURANPONG, Ph.D., CO-ADVISOR : PROF APIWAT MUTIRANGURA, Ph.D., 105 pp.

Background: Under physiological conditions, leukocytes contribute to the majority of circulating DNA in plasma. Therefore, detection of SHP-1 promoter-2 (SHP1P2) methylation to represent epithelial tumor-derived circulating nucleic acid may serve as a potential plasma biomarker for epithelial-derived cancer. Furthermore the fraction of cancer cell-derived and inflammatory cell-derived nucleic acid was still enigmatic. Total amount of circulating nucleic acid was increased in cancer patient and it was correlated with prognostic outcome in various types of cancer. These might be the effect of tumor-derived nucleic acid, correlated with tumor burden; however the interaction of cancer and immune cell was not negligent.

Materials and method: A dual hybridization probe and real-time quantitative PCR-based assay was used to determine the level of SHP1P2 methylation in plasma. Blood samples were prospectively collected from 58 advanced NSCLC patients and 52 healthy control. Clinicopathological data and outcome of treatment were included in the analysis.

Results: The levels of SHP1P2 methylation in plasma from controls were mostly undetectable. In contrast to the NSCLC patients, SHP1P2 methylation was readily detectable with a median of 0.77 ng ml^{-1} [0–26.5 ng ml^{-1}], which was significantly higher than that of controls. Pretreatment level of the SHP1P2 methylation was significant associated with the survival. Patients who had SHP1P2 methylation < 0.7 ng ml^{-1} had better progression-free survival (5.2 vs. 2.6 months, p = 0.009) and overall survival (12.6 vs. 7.6 months, p = 0.01). SHP1P2 methylation was the only independent predictive factor of survival by multivariate analysis. Moreover circulating DNA level was also correlated with survival outcome however less specific than SHP1P2 methylation. The follow-up level correlated well with response of treatment. This influenced by the impact of tumor-derived and inflammatory cell-derived nucleic acid.

Conclusion: Measurement plasma SHP1P2 methylation may serve as a potential non-invasive biomarker for the diagnosis and prognosis assessment in lung cancer patients adding to circulating DNA level. This classification may serve as a biomarker for risk-adaptive treatment

Field of Study : Biomedical Sciences Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

Co-advisor's Signature .....

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CONTENTS

Page

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

PAGE

LIST OF ABBREVIATIONS

NSCLC	Non-small cell lung cancer
SHP1P2 MI	SHP-1 promoter 2 methylation index
mSHP1P2	Methylated SHP-1 promoter 2 status
uSHP1P2	Unmethylated SHP-1 promoter 2 status
AQAMA	Absolute quantitative version of quantitative assessment of methylated alleles ARMS amplification-refractory mutation system
MSP-PCR	Methylation specific polymerase reaction
OS	Overall survival
PFS	Progression-free survival
CNAPS	Circulating nucleic acid in plasma or serum
EBV	*Epstein-Barr virus*
NPC	Nasopharyngeal cancer
hTERT	Human telomerase reverse transcriptase
FHIT	Fragile histidine triad
APC	Adenomatous polyposis coli
CDH13	Cadherin 13
RAR β	Retinoic acid receptor beta
RASSF1A	Ras association domain family 1
DAPK	Death-associated protein kinase
GSTP1	Glutathione S-transferase P1
MGMT	-methylguanine-DNA-methyltransferase
NK/T	Natural killer/ T-cell lymphoma
ATLL	Acute T-cell lymphoblastic leukemia
CSF-1	Colony-stimulating factor 1
EGF	Epidermal growth factor
Epo-R	Erythropoietin receptor
IFNa/b-R	Interferon alpha/beta receptor

IL	Interleukin
TCR	T-cell receptor
STAT	Signal transducers and activators of transcription
RECIST	Response evaluation criteria in solid tumor
ECOG	Eastern cooperative oncology group

CHAPTER I

INTRODUCTION

1.1. RATIONAL AND BACKGROUND OF CIRCULATING NUCLEIC ACID IN PLASMA OR SERUM (CNAPS)

1.1.1. BIOLOGY OF CIRCULATING NUCLEIC ACID IN HEALTHY ADULT AND VARIOUS PATHOLOGICAL CONDITIONS

The study of circulating DNA in plasma and serum of healthy adult has been conducted since 1975 by Steinman C.R. et al.[1] Since then the mechanisms by which DNA is released into serum or plasma are still enigmatic. The possibility of DNA release might be from apoptosis, necrosis or active secretion by certain cell populations. [2-6] Even though we don't know the exact mechanism, some studies showed importance biological activity of released DNA by incorporated genome into nucleus and expressed its function. [7-10] Adams D.H. et al. reported the in vitro interaction of immune system with tumor-derived nucleic acid. The increased DNA synthesis was resulted from transmit genetic messaged to immune cell.[11]

The biology of circulating nucleic acid in plasma and serum of healthy individuals is predominantly hematopoietic in origin has been conducted by Lui, Y.Y. et al. In sex-mismatched bone marrow transplantation model, approximate 60% of Y-chromosome DNA was detected in female patients plasma who receiving bone marrow transplantation from male donor. [12]

Elevated amount of plasma/serum circulating DNA higher than healthy volunteer was found in several pathological conditions such as systemic lupus erythematosus, sepsis, trauma, pulmonary embolism, cardiovascular disease, stroke, various types of cancers and etc. It was also correlated well with active stage of disease [13-15]. Those studies concluded that circulating DNA level might have role of diagnosis or prognostic potential in various pathological conditions. However circulating nucleic acid is still not included into the routine molecular testing.

1.1.2. BIOLOGY OF MOLECULAR MARKER IN PLASMA OR SERUM OF CANCER PATIENT

1.1.2.1. THE IMPACT OF INTEGRITY OF CIRCULATING DNA IN CANCER

Tumor necrosis is a frequent event in malignancy. It generates a spectrum of DNA fragments with different strand lengths[16]. In contrast to cell death in normal tissues which is mainly apoptotic process resulted small and uniform DNA fragments. This phenomenon was established in several solid malignancies such as colon cancer, prostate cancer, breast cancer, ovarian cancer, endometrial cancer. The range of DNA fragment length used for evaluation DNA integrity was varying from 400 bps -1.8 kbps. [17-21]

Using semi quantitative real-time PCR of beta-actin gene with 100-bp and 400-bp PCR product resulted in relatively lower abundance of longer DNA fragments in plasma samples of 65 non neoplastic conditions but not in 61 breast and gynecologic cancer [19]. Similar result with schmidt B et al study using quantitative real-time PCR with hybridization probe targeting the single copy human endogenous retrovirus sequence (ERV) to assess plasma DNA level by amplified fragments length 135-bp, 419-bp and 618-bp. Even though this study didn't find significant difference of each fragment length DNA concentration in plasma and serum of lung cancer or benign lung disease patient, the author reported the association between the longer DNA fragments and the lower amount of DNA concentration [22]. ALU repeated DNA sequence, the short interspersed element which was the target of DNA integrity assessment in Umetani, N. *et al* study, was used to measure serum and plasma DNA integrity by quantitative real-time PCR using ratio of longer (247 bps) to shorter (115 bps) DNA fragment. The result was consistent with afore-mentioned studies , the longer fragmented DNA was correlated with the lower amount of DNA concentration [20].

Apoptosis process in normal tissue result fragmented DNA approximately 185-200 bps. Suzuki N et al study explored the biology of circulating DNA in healthy volunteer. Purified DNA from plasma sample was cloned and sequenced independently. The mean value of circulating DNA in this study was 176 bps, range 61-567 bps, 65% of the fragments was 151-180 bps in length with was the most frequent length between 161-170 bps. This fragmented DNA was originated from various loci of chromosomes [16, 23]. Moreover Jahr S et al study was found that the

mostly frequently observed the DNA fragment length in plasma of cancer patient was 180 bps, some accompanies by high-molecular-weight DNA fragment larger than 10000 bps. [4] The difference nature of plasma DNA integrity in cancer and healthy might influenced the result of the experiment

1.1.2.2. THE CLINICAL IMPLICATION OF MOLECULAR MARKER IN CANCER PATIENT

The potentially implication in clinical practice of CNAPS in cancer patient was speculated in the aspect of prognostic factor or therapy monitoring during follow-up due to nonspecific elevated level in many pathological condition. At least 4 studies in showed the implication of baseline circulating DNA level as a prognosis factor and therapy monitoring in lung, breast, prostate and colon cancer. [24-27]

The presence of tumor-derived DNA in plasma/serum of cancer patient as the component of circulating DNA level had been proved since Sorenson et al. and vasioukhin et al. [28, 29] Later on several studies explored the detection of tumor-specific molecular marker in cancer patients were conducted. Jahr S et al. Using quantitative methylation-specific PCR of the promoter region of CDKN2A tumor suppressor gene, show that circulating nucleic acid is composed of tumor-derived and non tumor-derived circulating nucleic acid. The fraction of tumor-derived nucleic acid was range from 3-93% of total circulating DNA in 11 from 25 (44%) detectable CDKN2A methylation of unselected type and various stage cancer patients. The experiment could not conclude the origin of non tumor-derived nucleic acid and magnitude but at least Jahr S et al study reported the negative correlation between non tumor-derived portion, tumor- infiltrate T lymphocyte and angiogenesis. [4]

The establishment of specific tumor-derived molecular marker with good sensitivity and specificity represents the ultimate target for clinical usefulness in cancer management. Several molecular markers such as genetic alteration, epigenetic alteration, microsatellite marker, viral DNA, mitochondrial DNA and cell-free mRNA in plasma/serum have been conducted in many studies included various types of cancer patient. Viral DNA detection, the most progressive biomarker, had been used as molecular biomarker in viral-associated cancers such as EBV DNA in nasopharyngeal cancer (NPC) patient and HPV DNA in cervical cancer. Detectable EBV DNA in untreated NPC but not healthy volunteer [30] and before obvious

disease relapsed was first reported in 6 NPC patients whereas undetectable EBV DNA in 11 patient who remained in remission.[31] Validate study was conducted in a cohort with 99 NPC patients and it confirmed the clinical usefulness of EBV DNA in potential diagnostic tool, disease monitoring and predict outcome of treatment. [32] HPV DNA also was detected in proportion of cervical cancer patient.[33] Those are the examples of CNAPS confirmed by many studies about their role of potential prognostic marker and monitoring of disease recurrence. Currently pretreatment EBV DNA load is incorporated as prognostic factor to International Union Against Cancer (UICC) Staging in NPC.[34]

1.1.3. MOLECULAR MARKER IN PLASMA OR SERUM OF NON-SMALL CELL LUNG CANCER AND CLINICAL APPLICATION

In this thesis we focus on non-small cell lung cancer (NSCLC) which is the crucial disease and the leading cause of death in the world even progressive of novel treatment strategy [35, 36]. Numerous molecular markers have been studied aim to improved outcome of this lethal disease. We mentioned previous studies in here.

1.1.3.1. CIRCULATING DNA LEVELS

Circulating DNA level is one of the molecular markers which initially postulates as diagnostic molecular marker by Sozzi,G et al study. Using real-time quantitative polymerase chain reaction amplification of human telomerase reverse transcriptase gene (hTERT) in plasma of pretreatment stage I-IV NSCLC compare with healthy volunteer control, the significant higher levels of circulating DNA level in NSCLC than control were found. Follow-up circulating DNA level after definitive treatment (surgery or chemoradiation) had diagnostic potential of disease relapsed. [37] However owing to elevated circulating nucleic acid was found in various pathological conditions which might interfere accuracy of this molecular marker. Subsequent Gautschi O et al study explored role of circulating DNA as the prognostic marker in NSCLC patient. Elevated amount of circulating DNA by real-time quantitative polymerase chain reaction amplification of GAPDH gene before treatment in 185 various stage patients was correlated well with poor prognosis. However incorporate clinicopathological data by multivariate analysis found that not

only elevated amount of circulating DNA level but also age ≥ 70 yrs and stage IV disease were also correlated with decreased survival. Increasing level of circulating DNA level in stage IV NSCLC after received chemotherapy treatment was associated with disease progression.[24] These studies postulated the implication of circulating DNA level as prognostic biomarker in NSCLC.

1.1.3.2. TUMOR-DERIVED SPECIFIC MOLECULAR MARKER

Many cancer-specific molecular markers in plasma/serum of NSCLC have been studied to discover an ultimate target, a good sensitivity and specificity marker for early diagnosis and more effective management. Common genetic alteration or molecular pathogenesis of lung cancer is preferable the target of those biomarker studies.

1.1.3.2.1. MICROSATELLITE INSTABILITY

The present of allelic imbalance at loci on 3p (fragile histidine triad [FHIT]) is one of the common molecular markers in NSCLC which had been studied by Sozzi G et al. The detection rate of microsatellite instability and LOH in primary tumor specimen of stage I-III NSCLC was 61% (20/33) while the concordance plasma/tumor microsatellite instability/LOH was 45%. (9/20) [38] Similar detection rate of microsatellite alteration FHIT locus was found in primary tumor specimen by Francesca Andriani F et al. study included 64 resectable NSCLC. This study also combined another two molecular markers, microsatellite alteration at 3p other than FHIT loci and p53 mutation. Using at least 1 of 3 molecular markers, the plasma detection rate in all stage was 51.6%. [39]

1.1.3.2.2. EPIGENETIC ALTERATION OF TUMOR SUPPRESSOR GENES

Epigenetic alteration of tumor suppressor genes in human cancer is the common feature of cancer and it is considered as the important molecular markers.[40] Several studies have shown that tumor-specific epigenetic alterations could be detected in DNA recovered from plasma or serum of NSCLC patients.

Aberrant APC promoter 1 methylation was detected in 95 of 99 (96%) of resectable lung cancer. Preoperative serum or plasma sample was analyzed by quantitative methylation-specific fluorogenic real-time PCR with detected rate 47% (42 of 89 patients). Median level of circulating methylated APC promoter 1 fraction was 0.36 (range 0.01-11.5). Detectable rate was different in pair plasma (14 of 15, 93%) and serum (6 of 15, 40%). No methylated APC promoter DNA was detected in serum sample form 50 healthy controls. Elevated APC methylation level in lung cancer tissue but not plasma/serum was independent factor predicting inferior survival. The logistic regression model was done include clinicopathological parameters. [41]

Table 1.1 The prevalence of 4 epigenetic markers for the detection of lung cancer cells in serum of stage I-III non-small cell lung cancer matched primary tumor and serum [42]

Gene aberrantly methylated	Occurrence in tumor no./total no. (%)	Serum positive no./total no. (%)
p16	9/22 (41)	3/9 (33)
DAPkinase	5/22 (23)	4/5 (80)
GSTP1	2/22 (9)	1/2 (50)
MGMT	6/22 (27)	4/6 (66)

Several aberrant DNA methylation such as p16, death-associated protein kinase, glutathione S-transferase P1 and DNA repair gene -methylguanine-DNA-methyltransferase have been studied in match serum/tissue sample of various stage of resectable non-small cell lung cancer. The prevalence of aberrant DNA methylation was 41%, 23%, 9%, and 27% in primary tumor. The detection rate of correlate serum aberrant DNA methylation was 33%, 80%, 50% and 66% in those groups with aberrant DNA methylation in primary tumor. This represents only 50% sensitivity detection rate in whole population with combine aberrant DNA methylation marker.[42]

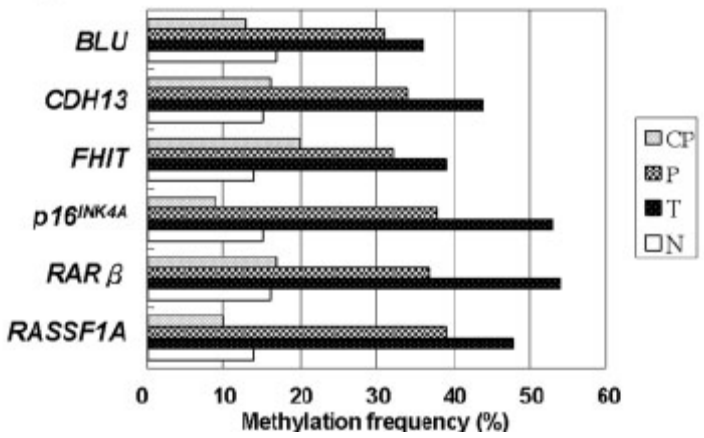


Figure 1.1 The prevalence of 6 aberrant DNA methylation genes in stage I-III non-small cell lung cancer matched normal lung/primary tumor and plasma of individual patient with sex/age/smoking status matched control [43]

The later study used 6 aberrant DNA methylation markers include tumor suppressor gene (BLU), cadherin 13 gene (CDH13), the fragile histidine triad gene (FHIT), the cell cycle control gene p16, the retinoic acid receptor β gene(RAR β)and the Ras association domain family 1 gene (RASSF1A). Sixty-three matched primary tumor/plasma sample stage I-III non-small cell lung cancer and 36 age/sex/smoking status match cancer-free control were analyzed. The prevalence of those aberrant DNA methylation in non-small cell lung cancer patient were 36%, 44%, 39%, 53%, 54% and 48%. Even though high concordance of methylation status of each gene between tumor and corresponding plasma samples 75-86%, overall yield when use at least 2 aberrant DNA methylation marker presented in serum was only 63%. The prevalence of aberrant DNA methylation in control group were 13%, 16%, 20%, 9%, 17% and 10% respectively without cancer diagnosis in 2 year follow-up period after aberrant DNA methylation was detected. This might lead to false positive result when use this aberrant DNA methylation as a diagnosis tool for non-small cell lung cancer. [43]

1.1.3.2.3. CLINICAL APPLICATION OF MOLECULAR MARKER IN NON-SMALL CELL LUNG CANCER

The major limiting steps for a routine use of molecular markers detection of plasma or serum in clinical practice were lack of the sensitive and specific testing. No

strongly evidence of plasma biomarker in non-small cell lung cancer which had valuable useful in clinical practice. Most studies use panel of molecular markers and described the overall positivity rate as a sensitivity rate which is might not correct. Moreover no combination of markers has yet been shown to be the prognostic value. An advancement of the technique, sensitive method and quantitative manner, the studies in this field had been expected to be an important development field of genetic testing and molecular diagnostics. The establishment of specific molecular markers detecting 100% of lung cancer and good discrimination with healthy individual undoubtedly represents the ultimate target. By this reason the concept of using tissue specific methylation marker as novel biomarker of prognostic evaluation and explore biology of plasma circulating DNA of non-small cell lung cancer is a challenging research question to prove principle concept. The combination of quantitative approach on plasma DNA will improve the non-invasive testing to lung cancer evaluation.

1.2. RATIONAL AND BACKGROUND OF POTENTIAL BIOMARKER ABERRANT SHP-1 PROMOTER 2 METHYLATION

1.2.1. THE CHARACTERISTIC OF SHP-1 GENE

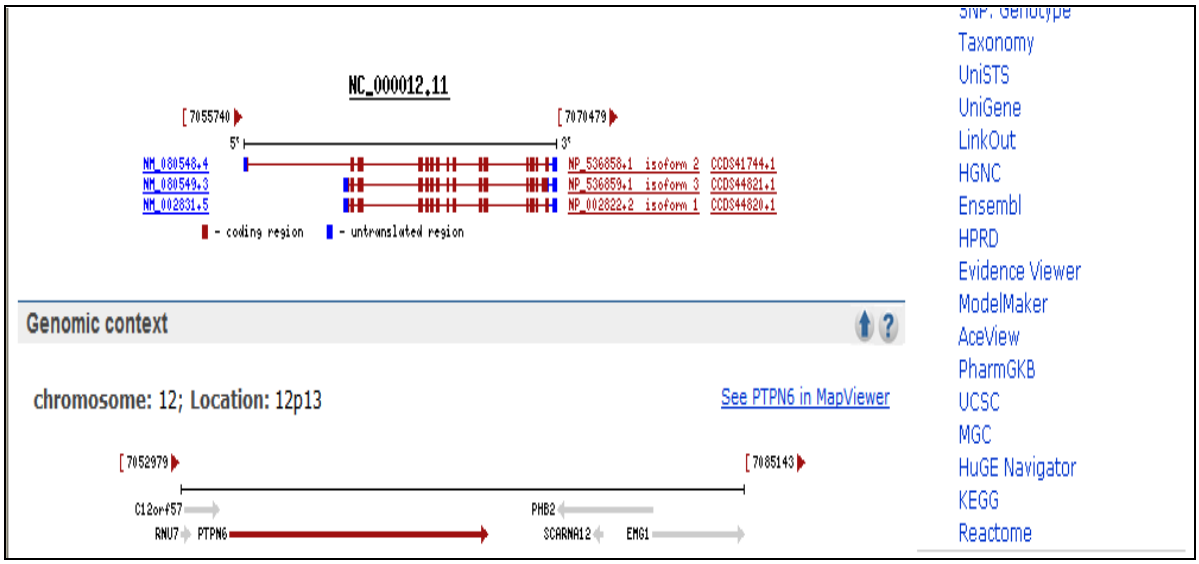
SHP-1 or PTPN6, an intracellular protein tyrosine phosphatase composes of two N-terminal Src homology-2 domains that allow binding to phosphotyrosines residues, was located on chromosome 12. It consists of 17 exons spanning 17 kb of DNA. Banville D et al. study identified 2 transcription initiation sites of SHP-1 gene by the alternate splicing and exon skipping. These mechanisms result of 2 difference transcriptions size, 2.4 and 2.6 kb, difference at their N-termini. The two translation initiation of epithelial cell and hematopoietic cell isoforms of PTPN6 are located within exon 1 and 2 respectively. The pattern of transcription is determined by usage of either of 2 tissue-specific promoter sequences. Promoter 1 located approximated 7 kb upstream from the second promoter, is active in all cells of epithelial cell origins but not active in hematopoietic lineage. Conversely promoter 2 is active in cell of hematopoietic lineage but not epithelial cell. [44]

Other characteristic of this gene, exon 3 and 4 encode the N-terminal SH2 domain, while exon 5 and 6 encode the C-terminal SH2 domain. Exon 7 encodes the sequence connecting SH2 domains to the catalytic domain of PTPN6. This region includes the peptide sequence of GFWEEFE that is conserved in the mouse and human. Exon 8-14 encodes catalytic domain of PTPN6. The translation stop codon is located within exon 16.

1.2.2. THE FUNCTIONA AND EXPRESSION OF PROTEIN TYROSINE PHOSPHATASE SHP-1

SHP-1 has been shown to interact with a diverse range of cytosolic and membrane-bound signaling protein. It has distinct role in signaling processes depend on cell type, as negative regulator of cellular transduction in hematopoietic cells whereas positive regulator in nonhematopoietic cells.

SHP-1 has been previously considered as a tumor suppressor gene in hematopoietic cancer due to high-frequent silencing by promoter methylation or post-transcriptional modification in various types of hematopoietic malignancy especially highly aggressive lymphomas such as NK/T, ATLL and Burkitt's lymphomas. [45-49] The function of SHP-1 in hematopoietic cell has been explored by several studies. It has been projected as a negative regulator for intracellular signaling of cell proliferation and differentiation of hematopoietic cells via transmembrane receptors: growth factor receptors with an intrinsic tyrosine kinase activity (i.e. c-kit, CSF-1, TrkA and EGF) [50-55] cytokine receptors (e.g. Epo-R, IFNa/b-R, IL-3R and IL-2R) [56-59] and receptors involved in the immune response such as the TCR complex and CD5 [60, 61]



://www.ncbi.

Figure 1.2 SHP-1 gene location and transcription patterns

Conversely, SHP-1 expression was found in various type of epithelial cancer cell line such as breast cancer[62], ovarian cancer[63], pancreatic cancer [64] and prostate cancer[65] The distinct function of SHP-1 gene was found in epithelial cell. Su et al studied the positive effect of SHP-1 in epidermal growth factor via mitogenic signaling pathway. Overexpression of a catalytically inactive mutant of SHP-1 in HEK293 cells strongly suppress mitogen-activated pathways and results in decrease cell growth, DNA synthesis and the transcription of early response genes. [66] Consistent with You M et al studied, transfection of Hela cells with inactive SHP-1 reduced the signal transducer and activator of transcription (STAT)-DNA binding induced by interferon γ and epidermal growth factor. Furthermore overexpression of SHP-1 enhanced activity EGF-induced activation of the mitogen-activited protein kinase pathway [67].

Cragg G et al studied shown the distinctly difference localization of SHP-1 between hematopoietic and epithelial cell. Therefore SHP-1 was distributed in cytoplasm of hematopoietic cell, with very little nuclear SHP-1 evident while it was nuclear localization in epithelial cells [68]. Even though the molecular basis for apparent opposite effects of SHP-1 in different cell systems has yet to be defined. Distinct expression of SHP-1 isoform in hematopoietic cancer was subsequent revealed. Loss of SHP-1 isoform 1 expression, mainly due to promoter 1 hypermethylation by Takashi O et al study [47, 69]. Contrast to vigorous expression

of SHP-1 isoform 2 by Denis B et al study. [44] There might be difference function of 2 distinct SHP-1 transcriptions using distinct 2 promoter in hematopoietic and epithelial cell.

1.2.3. POTENTIAL ROLE OF ABERRANT SHP-1 PROMOTER 2 METHYLATION AS A NOVEL PLASMA BIOMARKER

The distinct role and pattern of SHP-1 in hematopoietic and epithelial cell was further explored by Ruchusatsawat, K. et al study [70] which identified SHP-1 promoter 2 as a tissue-specific methylation regulation, control by distinct promoter hypermethylation, and involved with pathological epithelial condition other than malignancy. SHP-1 promoter 2 complete methylation was constantly detected in all epithelial cell lines (Hela, Hep2, SW480, HaCaT and HepG2) and normal epithelium from several organs such as prostate gland, breast, lung, kidney and liver whereas in normal hematopoietic cell without exception was nonmethylated. This study also confirmed the strong association between methylation of promoters and their activities.

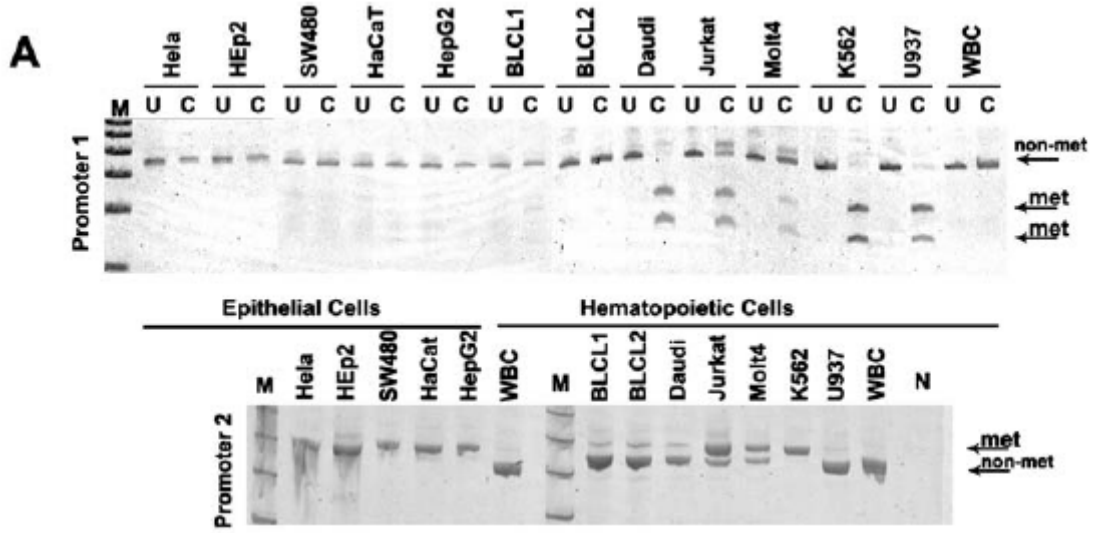


Figure 1.3 Promoter 1 and 2 methylation patterns in various epithelial, hematopoietic cell lines and normal WBC [70]

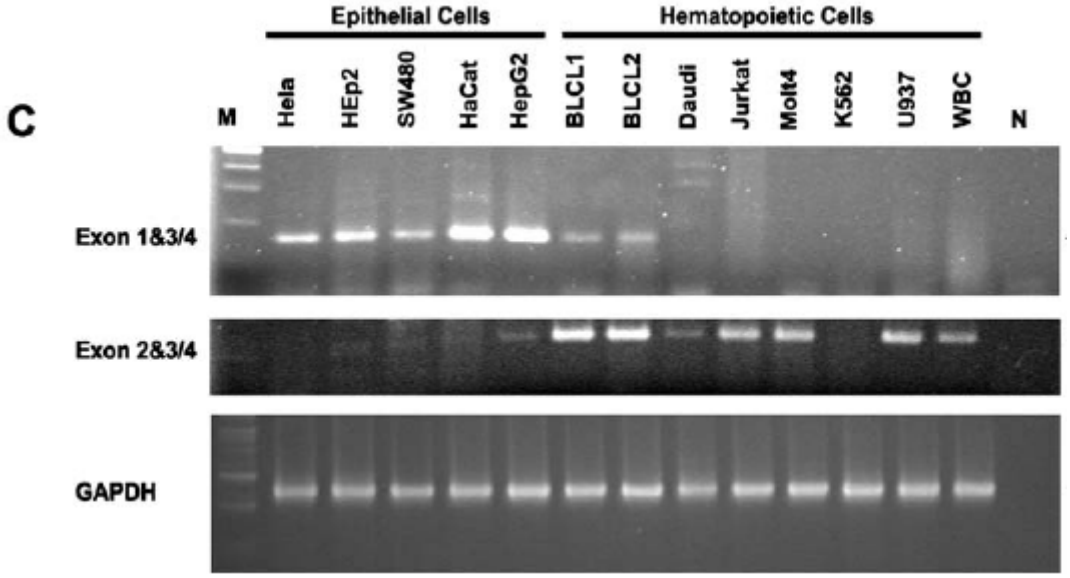


Figure 1.4 SHP- 1 isoform 1 and 2 expression of various epithelial, hematopoietic cell lines and normal WBC [70]

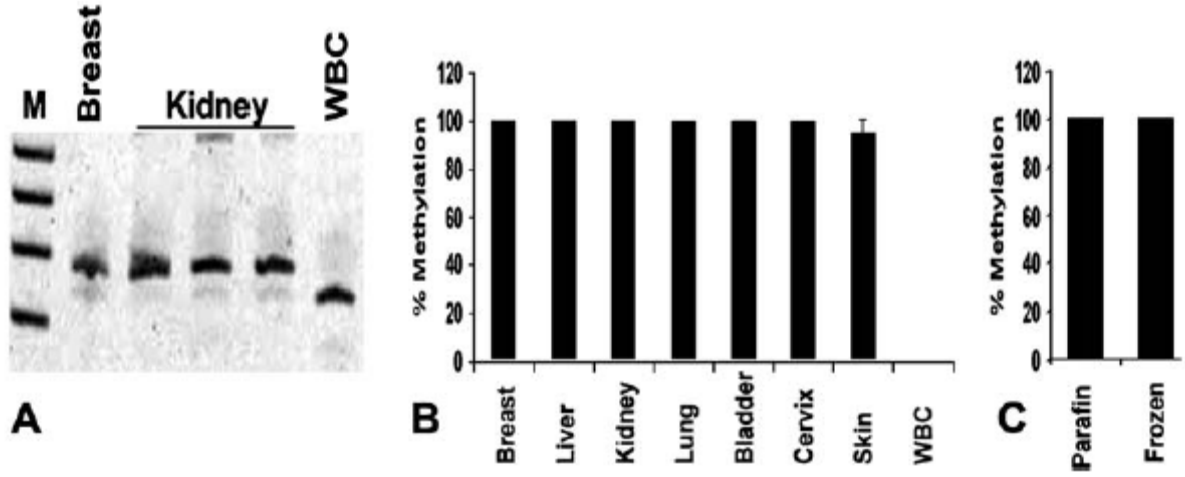


Figure 1.5 SHP-1 promoter 2 methylation in epithelium A. representative epithelial tissue from microdissected, paraffin-embedded tissue of breast, kidney and WBC B. representative average level of SHP-1 prometer 2 methylation status in epithelial tissue of several organs C. representative SHP-1 promoter 2 methylation in frozen and paraffin-embedded tissues of epithelia [70]

1.3. OUTLINE OF THE STUDY

1.3.1. RESEARCH QUESTION

In the condition that have predominantly hematopoietic cell source like plasma or serum in healthy adult, detecting SHP1P2 methylation could reflect abnormal epithelial cell source which is pathological condition. Many epithelial cancers such as breast, lung or colon cancer which previous study explored tumor-derived DNA in plasma or serum is an interesting model to prove the principle of concept that SHP1P2 methylation could be a novel biomarker for cancer detection.

Furthermore, combination fraction of SHP1P2 methylation or unmethylation could reveal the biology of circulating DNA in malignancy, whether the correlation among tumor-derived nucleic acid, inflammatory cell-derived nucleic acid, demographic data and clinical outcome.

1.3.2. HYPOTHESIS

1.3.2.1. SHP1P2 methylation, which is epithelial specific methylation, might has clinical significant to detect tumor-derived circulating nucleic acid in plasma of advanced NSCLC compare with age/sex match healthy volunteer.

1.3.2.2. SHP1P2 methylation might be predictive marker of survival and progression of disease in advanced NSCLC.

1.3.2.3. The fraction of SHP1P2 methylation and unmethylation might difference in individual patient however there might be some correlation between those proportions and the clinical outcome.

1.3.3. OBJECTIVE OF THE STUDY

1.3.3.1. To prove specificity and sensitivity of SHP1P2 methylation status in plasma of advanced NSCLC but not healthy adult.

1.3.3.2. To prove that the level of SHP1P2 methylation status predict survival and progression of disease in chemotherapy treated advanced NSCLC.

1.3.3.3. To explore the fraction of circulating tumor-derived or inflammatory cell-derived nucleic acid in advanced NSCLC.

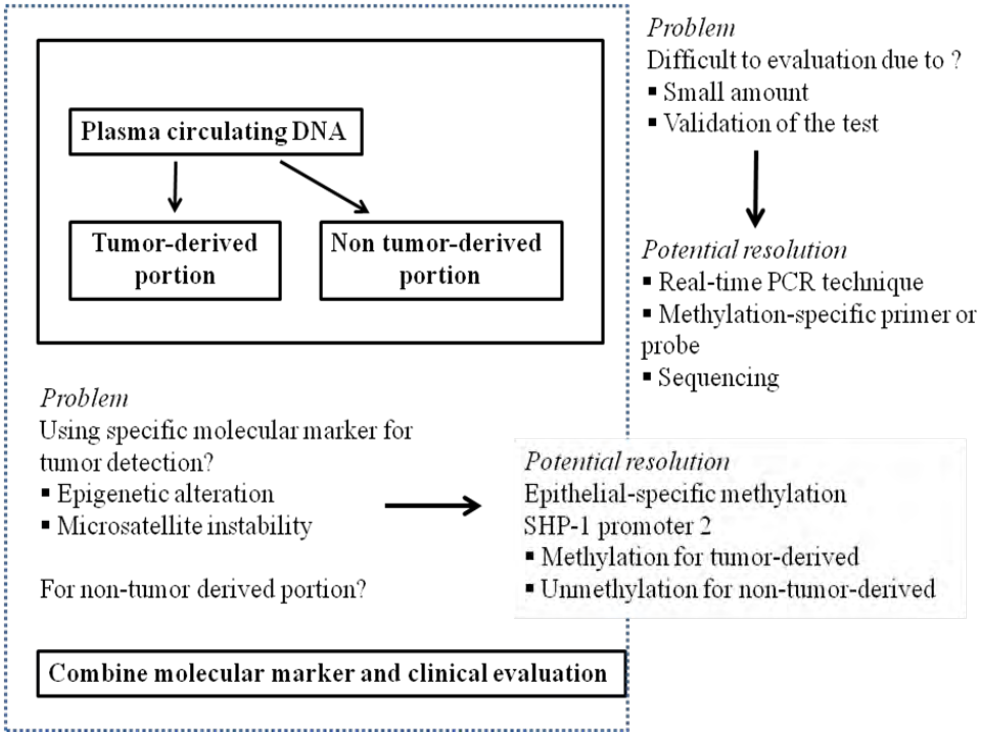


Figure 1.6 Conceptual framework of the study protocol (1)

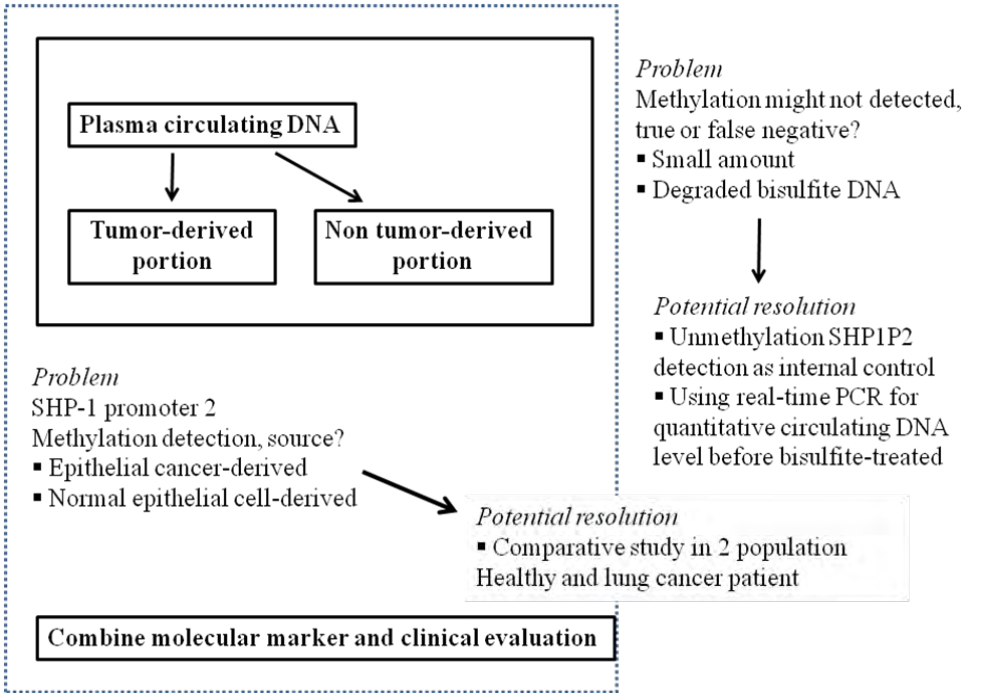


Figure 1.7 Conceptual framework of the study protocol (2)

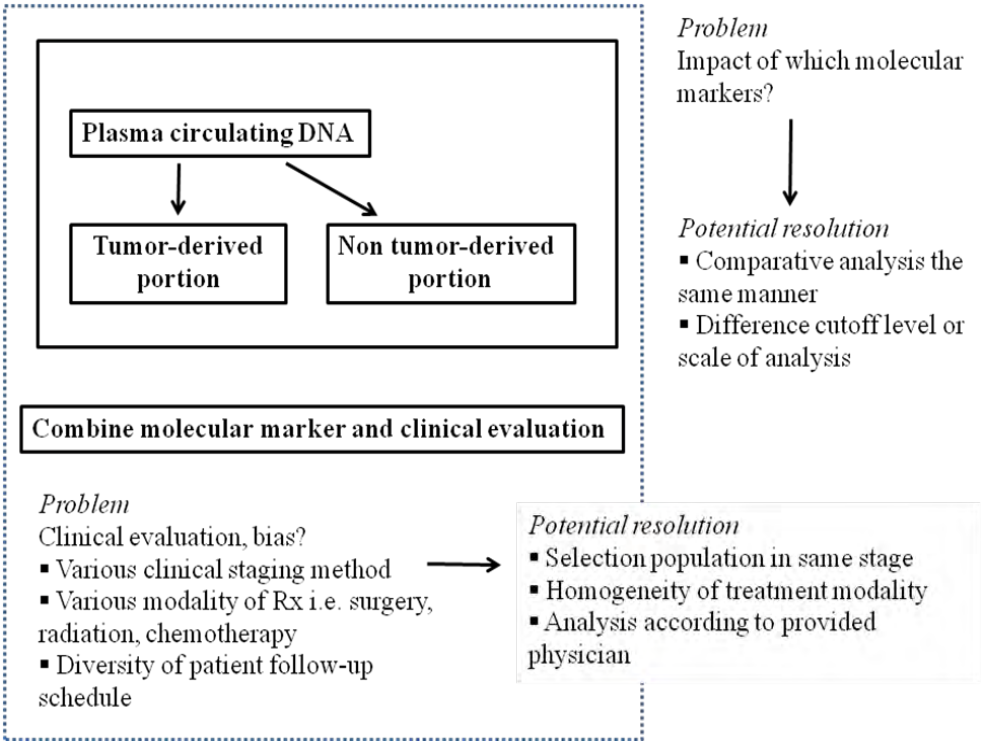


Figure 1.8 Conceptual framework of the study protocol (3)

CHAPTER II

MATERIALS AND METHODS

2.1. STUDY PARTICIPANTS

Module I: Blood sample collection was done from 2 difference target population. First, thirty-eight advanced NSCLC patients (stage IV and IIIB with malignant pleural effusion) who received treatment at medical oncology unit, The King Chulalongkorn Memorial Hospital (Bangkok,Thailand). In that time non-small cell lung cancer staging was done followed AJCC lung cancer staging. The second population was healthy volunteers who randomly gathering blood sample from a community in Bangkok. Non-smoking was included in a criterion of healthy volunteer to avoid occult medical condition. We selected all 52-sex and age match plasma sample of healthy volunteer with advanced NSCLC to further analyzed SHP-1 promoter 2 methylation status.

Module II: After the available data from first cohort, the number of NSCLC study participant was increased up to the total of fifty-eight in the period between September 2008-2009. All of those study participants was received first-line chemotherapy treatment and recruited into this study as prospective manner. Every patient had histopathological result to confirm the diagnosis and limit Eastern Cooperative Oncology Group (ECOG) scores for performance status of 0 to 2. The clinicopathological data of the patient at initial diagnosis and treatment implication along follow-up period was collected. The treatment decision, clinical assessment, baseline and interval imaging were done depend on provided physician. The data was censored at the end of study period in the remaining case with no disease progression or alive. The institutional review board approved the study protocol, and the entire patient provided written informed consent.

2.2. SELECTION OF BLOOD COMPONENT FOR ANALYSIS

Tumor-derived circulating nucleic acid was introduced as a potential noninvasive tool for cancer detection or therapy monitoring in cancer patients. Nevertheless the higher concentration of circulating nucleic acid was obtained in serum than plasma sample, the author chose plasma instead of serum to avoid interfering effect of cell lyses and genomic DNA releasing from leukocyte during clotting process from time-dependent sample preparation [12, 71]. Beside that Usadel, H et al study reported significant aberrant APC methylation detection in plasma than serum of non-small cell lung cancer patient (14/15 vs 6/15) [41]. The higher portion of circulating nucleic acid form leukocyte might interfere the ability of assay to detect tumor-derived nucleic acid.

2.3. SAMPLE COLLECTION

To obtain plasma sample, blood specimens from each patient at pretreatment chemotherapy, 3-week interval after first chemotherapy treatment and healthy volunteer had been collected in EDTA-containing tubes. Plasma samples were processed 2 rounds of centrifugation, first centrifuged at 1600 g for 10 minutes and then second centrifuged at 16000 g for 10 minutes. The plasma was stored at -80 degree Celsius before further analysis.

2.4. PLASMA DNA PREPARATION

Plasma DNA was extracted from 0.5-2 ml plasma sample using QIAamp blood minikit (Qiagen, Hilden, Germany) with ‘blood and body fluid protocol’ according to the manufacturer’s recommendations. Plasma was applied to each column and eluted into AE buffer 200 µl, repeated ethanol precipitation was done with final volume 20 µl to yield optimal concentration for further analysis.

2.5. QUANTITATIVE ANALYSIS OF CIRCULATING DNA LEVEL

Circulating DNA level was evaluated in all recruited plasma sample by using real-time PCR with dual hybridization probe specific sequence for GAPDH (NC_000012). The diversity of plasma DNA integrity between healthy and non-small cell lung cancer was also meditated at the time of setting up the experiment. The author referenced Suzuki N et al study which explored the biology of circulating

DNA in healthy volunteer. The mean value of circulating DNA was 176 bps, range 61-567 bps [16]. Moreover Jahr S et al study was found that the mostly frequently observed the DNA fragment length in plasma of cancer patient was 180 bps. [4] The established primers were conducted to amplify majority of circulating nucleic acid in the range of both populations.

The sequences of forward and reverse primers were 5'- CTA-CTC-TTT-CCA-CCC-TCG-GAG-T-3'and 5' TGA-GGC-CCT-GCA-GCG-TAC-TC-3' which represented 158-bp fragment length PCR product of GAPDH. The dual hybridization probe sequence was TAMRA-5'-TTG-GGG-TCC-CAA-CGT-GCG-TCG-3'-VIC. To generate standard curve, difference concentrations of control reference form 10, 1, 0.1, 0.01 ng/μl were prepared form normal control WBCs by 10-fold serial dilution. PCR was performed in 96-well reaction plate in duplication with final volume 20 μl. 2 μl 10x buffer, 0.4 mM of dNTPs, 0.2 mM of , 0.2 μM of each probe and primer, 0.2 μl of hotstar Taq Qiagen and 1 μl of plasma DNA. PCR condition was initial denaturation step at 95 C for 10 min, followed by 60 cycles of denaturation at 95 C for 10 sec, annealing at 58 C for 20 sec and extension 72 C for 30 sec. Standard amplification curve of DNA at a known quantity as previous mention was interpolate in every reaction with multiple blank to ensure no cross contamination between reactions. Real time PCR was performed by ABI Prism, 7700, Sequence Detection System. The threshold cycle of unknown target sample was obtained with relative amount of DNA in the experimental sample. The analysis of circulating DNA level was adjusted by volume of extracted plasma using the following equation

Equation: $C=Q*V_{DNA}/V_{PCR}*V_{ext}$

C represents the target concentration in plasma (ng ml^{-1}), *Q* represents the target quantity (ng) determined by a sequence detector by PCR, represents the total volume of DNA obtained after extraction, represents the volume of DNA used for PCR (1 μl) and represents the volume of plasma extraction.

2.6. BISULFITE DNA PREPARATION

Even though a number of methods have been developed to detect or quantify DNA methylation, the most common technique used today remains the bisulfite

conversion method. This technique converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. The bisulfite conversion method introduces various DNA strand breaks and results in highly fragmented single strand DNA. The degradation of DNA has been reported range between 84-96% [72]. The optimize bisulfite treatment by balancing competing goals of maintaining complete cytosine conversion and minimal DNA fragmentation was established. Bisulfite-treated DNA was conducted using EZ DNA Methylation- kit (Zymo Research, orange, CA, USA) that combines bisulfite conversion and DNA clean up. The kit follows a protocol from Paulin et al. [73] The protocol recommended the optimal amount of 200-500 ng DNA per bisulfite treatment due to awareness of incomplete bisulfite conversion. The lowest recommended amount of DNA per bisulfite treatment was 500pg. After plasma DNA extraction, concentration and measurement, the exact amount of circulating DNA from both groups was evaluated. Sodium bisulfite conversion of whole extracted plasma DNA but no more than 10 ng of circulating DNA, which covered majority amount of plasma DNA was selected by using the protocol recommended by the manufacturer.

2.7. ESTABLISH METHOD FOR SHP-1 PROMOTER 2 METHYLATION DETECTION

There are several molecular biology techniques for analyzing the methylation pattern in particular genes. Combine chemical modification such as methylation-sensitive restriction enzymes or sodium bisulfite DNA modification precedes PCR techniques or methylation-specific primer with PCR had been postulated [74]. Each method of methylation detection has potential clinical usefulness with distinctive method assessment.

Real-time polymerase chain reaction (PCR) is a novel method, commonly used for precise quantitative measurement of small amount of circulating nucleic acid. Combining specific fluorescence-detector dual hybridization probe (methylight) increases specificity of signal detection [75]. Moreover diversity of specific methylation detection was published such as methylation-specific real-time (MSP) PCR or combine using methylation-sensitive restriction enzyme digestion with real-time PCR. The author compared various method of detection in here. All SHP-1

oligonucleotide sequences were derived from GenBank (accession number U47924.1).

2.7.1. DETECTION OF SHP-1 PROMOTER 2 METHYLATION BY AMPLIFICATION-REFRACTORY MUTATION SYSTEM (ARMS) PRIMER

The amplification-refractory mutation system (ARMS) or PCR amplification of specific alleles is a simple and reliable method for detecting any mutation involving single base changes or small deletion. This technique is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample and will not amplify or minimizing false priming at the non target allele. The author applied this technique aim to amplify only methylated sequence. The 3`terminal base of methylated-specific ARMS primer was complemented to the methylated sequence but not unmethylated sequence. Additional deliberate mismatch was also introduced at the penultimate base of the ARMS primer to increase specificity of the ARMS reaction. Because different mismatches have been found to have different destabilizing effects, it is necessary to consider both terminal and penultimate mismatches together. If the mutation-induced mismatch is strong, a weak additional mismatch should be selected as indicated in Table 2.1. The oligonucleotide sequences were 5`-TGGAGGAGGGAGAGATGGC-3` and 5`AACACATATATACCTTAC ACACTCCAAA-`3. The sequences of methylated-specific ARMS forward primer comparative by methylated and unmethylated SHP1P2 sequences were shown in table 2.2. The author selected the penultimate mismatch followed the recommendation. The 82-bp PCR product was visualized by by gel electrophoresis on denaturing polyacrylamide gel and sybr green staining.

Table 2.1 Amplification-refractory mutation system primer additional mismatch selection

Terminal mismatch	Coding strand nucleotide corresponding to penultimate nucleotide in the primer			
	A	G	C	T
AA	A	G	A	G
AG	C	T	A	G
AC	G	A	C	T
TT	C	T	A	G
TG	G	A	G	C or T
TC	C	T	A	G
CC	C	T	A	G
GG	A	G	A	G

The red line represented mismatch selection for methylated primer in this study

Ref: Nicholas C. Dracopoli, Jonathan L. Haines, Bruce R. Korf, Cynthia C. Morton, Christine E. Seidman, J.G Seidman et al. Current protocols in human genetics volume 2 The United States of America: Wiley&Sons Inc, 2004.

Table 2.2 Amplification-refractory mutation system primer sequence for methylated SHP-1 promoter 2 detection

	Unmethylated sequence	Methylated sequence
Sequence	**5`-tggaggagggagagatg**T TGtgggaT-3`	**5`-tggaggagggagagatg**T CGtgggaTC-3`
Forward Primer	5`-tggaggagggagagatgg c-3`	5`-tggaggagggagagatgg c-3`

Note ; Red nucleotide represent corresponding 3`terminal nucleotide of ARMS primer, Green represent the penultimate nucleotide in the primer

2.7.2. DETECTION OF SHP-1 PROMOTER 2 BY HpaII METHYLATION-SENSITIVE RESTRICTION ENZYME

This is a classical method of methylation analysis based on the property of some restriction enzymes to be unable to cut methylated DNA. Since in eukaryotic DNA only cytosine in CG context can be methylated, classical common used enzymes pairs is HpaII-MspI which is recognized CCGG sequence (isoschizomer). HpaII is unable to cleaves DNA when the internal cytosine is methylated whereas MspI cleaves DNA irrespective of the presence of a methyl group at this position. This is the simpler and faster method to establish than bisulfite treatment; however it depended on available specific sequence for digestion. Using restriction enzyme resolved the problem of difficulty handling degraded DNA from bisulfite method. Moreover combining methylation-sensitive restriction enzyme digestion for detection and quantification of hypermethylated DNA sequences has shown good diagnostic sensitivity and specificity for detection serum hypermethylated RASSF1A sequences [76]. A hundred nanogram of isolate genomic DNA was digested overnight with 100 unit of HpaII enzyme at 37 degree Celsius in total volume 50 µl. The ratio of methylated-sensitive restriction enzyme and genomic DNA was followed previous report of detection hypermethylated RASSF1A in serum [76]. Inactivate the enzymes by heating at 65 degree Celsius for 20 minutes was done per manufacture protocol. PCR reaction contained 2 µl of digested genomic DNA and oligonucleotides were performed. The Oligonucleotide sequences were 5`- CTCTTCCTgTCCCCgCCCT -3` and 5`-TAAgCCTCAgATgCAgCTCCCAgT-3`. Control untreated restriction enzyme genomic DNA was ensured together in every treated genomic DNA reaction. The 114-bp PCR product was visualized by gel electrophoresis on denaturing polyacrylamide gel and sybr green staining.

2.7.3. DETECTION OF SHP-1 PROMOTER 2 METHYLATION BY METHYLATION-SPECIFIC PCR ASSAY

SHP1P2 methylation-specific PCR (MSP-PCR) primers and unmethylation-specific PCR (USP-PCR) primers which have been previously described identical both Ruchusatsawat K et al [70] and Koyama M et al [77] study have been used in here. The bisulfite sequencing determined CpG sites within SHP1P2 region was validate from both studies and its showed strong correlation between methylation signals from MSP or USP and sequencing. Duplex reaction containing bisulfite-treated DNA and specific oligonucleotide was performed. The methylated MSP

primers are 5′-TGT-GAA-CGT-TAT-TAT-AGT-ATA-GCG-3′ and 5′-CCA-AAT-AAT-ACT-TCA-CGC-ATA-CG-3′. The unmethylated MSP primers are 5′-GTG-AAT-GTT-ATT-ATA-GTA-TAG-TGT-TTG-G-3′ and 5′-TTC-ACA-CAT-ACA-AAC-CCA-AAC-AAT-3′. Two different sizes of the PCR products, 174 bp and 162 bp for methylated and unmethylated respectively were visualized by gel electrophoresis on denaturing polyacrylamide gel. The amplicons were analyzed by staining with sybr green. The SHP1P2 methylation index (MI) was calculated as a ratio of the intensity of the methylated amplicon divided by the sum of the methylated and unmethylated amplicon measured by a phosphoimager using the Image Quant Software.

2.7.4. QUANTITAIVE ANALYSIS OF SHP-1 PROMOTER 2 METHYLATION BY REAL-TIME PCR (METHYLIGHT)

This technique depends on a specific dual hybridization probe with specific reporter dye at the 5` end and quencher at the 3` end. The 5` to 3` nuclease activity of *Taq* DNA polymerase cut the probe and frees the reporter whose fluorescence is detected by a laser detector. The fluorescence is proportional to the number of copies of the amplified sequence. To perform the quantitative analysis of methylation status, there are three ways to differentiate between methylated and unmethylated template sequence: design methylation-specific primers containing or not CpG dinucleotides, design fluorescent labeled probe with one or several CpG sites or design both the above primers and probes. This method not only makes it possible to difference between methylated or unmethylated sequences but also defines the specific methylation patterns.

The PCR reaction was conducted in one reaction for assessment both methylated and unmethylated sequences to ensure a high efficiency of evaluation and decrease chance of PCR product contamination. The SHP-1 bisulfite forward and reverse primer sequences were 5’-GGT-GGA-GGA-GGG-AGA-GAT-GT -‘3 and 5’-AAC-ACA-TAT-ATA-CCT-TAC-ACA-CTC-CAA-A-‘3 . Methylation and unmethylation specific dual hybridization probe sequence were 5’-VIC-ACG-AAC-CCA-AAC-GAT-CCC-ACG-TAMRA-3’ and 5’-FAM-CAC-ATA-CAA-ACC-CAA-ACA-ATC-CCA-CA-TAMRA-3’ respectively. An absolute quantitative version of

QAMA was used to calculate methylation status [78]. To generate a standard curve, 10 ng DNA was prepared in different mixing ratios of methylated and unmethylated target sequences, hela and WBC respectively prior to bisulfite modification. The standard sample set was freshly bisulfited and included in every set of real time PCR. The following ratios were prepared (methylated/unmethylated): 0/100, 10/90, 25/75, 50/50, 75/25, 90/10 and 100/0.

Real-time PCR was performed in 96-well reaction plate in duplication fashion using ABI Prism, 7700, Sequence Detection System. The relative prevalence of either the methylated or the unmethylated allele was set to index equal 1 and 0 in the case that only one fluorescence signal crossed the threshold, indicating a relative absence of the opposite target. The 83 bps PCR products were visualized by 8% acrylamide gel electrophoresis and Sybr green staining to ensure specific amplicon. Multiple non-template controls were run with every assay to ensure no contamination across reaction. Equation:

Methylation index (MI) = methylated sequence percentage from AQAMA
mSHP1P2 = methylated sequence percentage from AQAMA * C
uSHP1P2 = unmethylated percentage from AQAMA * C

Where *C* represented the target concentration in plasma (ng ml^{-1}), *mSHP1P2* represented the absolute among of methylated SHP1P2 (ng ml^{-1}), and uSHP1P2 represented the absolute among of unmethylated SHP1P2 (ng ml^{-1}). AQAMA represented absolute quantitative version of QAMA.

These measured methylation index, absolute level of methylated SHP1P2, absolute level of unmethylated SHP1P2 and circulating DNA level parameters were further analyzed correlated with the clinical and pathological data.

2.8. STATISTICAL ANALYSIS

Mann-Whitney *U*-test was used to assess the difference between the non-parametric distributed variables. The data was expressed as median. Comparison between the groups was carried out by the or Fisher's exact test for categorical variables. The Spearman's correlation, two-sided test with 95% confidence interval

was used to assess the correlation between each variable. The receiver operative characteristic (ROC) curve analysis was done to evaluate the diagnostic potential of the SHP1P2 methylation. Furthermore, the sensitivity and specificity estimations for different thresholds of SHP1P2 methylation and AUC-ROC were carried out. The differences in the SHP1P2 methylation between baseline and follow-up samples were calculated using the Wilcoxon matched-pairs signed rank test. The significant level of $p \leq 0.01$ with a two-sided test was used. All statistical analyses were performed using SPSS version 16.0 (SPSS Inc, Chicago, IL, US).

For survival analysis, Kaplan-Meier estimates method was used. Progression-free survival (PFS) and overall survival (OS) were defined as an interval between the first date of chemotherapy treatment and the date of evidence of disease progression and death, respectively. The difference between the survival curves was compared by log-rank testing. Cox proportional hazards' regression analysis was used to estimate univariate and multivariate hazard ratios for both PFS and OS.

CHAPTER III

RESULTS

PART I SETTING UP THE SHP-1 PROMOTER 2 METHYLATION DETECTION

3.1. DETECTION OF SHP-1 PROMOTER 2 BY AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS) PRIMER

The 82-bp PCR product was expected from the ARMS primer and visualized by gel electrophoresis. The diverse annealing temperature PCR reaction range 50-58 degree Celsius was set up to decrease non specific PCR product. However PCR amplicon was still equally visualized in both control methylated and unmethylated sample, in here represented by Hela and WBC respectively, Figure 3.1. The designed ARMS primer didn't amplify only the methylated sequences as initially plan.

3.2. DETECTION OF SHP-1 PROMOTER 2 BY HpaII METHYLATION-SENSITIVE RESTRICTION ENZYME

The gel visualized 114-bp PCR amplicon was shown in Figure 3.2(A). The residual PCR amplicon from 16 hrs incubated Daudi cell line DNA with HpaII restriction enzyme was found. These might due to incomplete digestion of unmethylated sequences. Then the subsequences protocols included using 150 unit of HpaII enzyme per 100 ng of genomic DNA and prolong incubation period up to 24 hrs with afore-mentioned enzyme concentration were done. The effective protocol was the former which increased amount of HpaII restriction enzyme. No residual target PCR amplicon in Daudi cell line DNA was shown by this protocol, Figure 3.2(B) however the increasing HpaII enzyme protocol also affected the level of methylated SHP1P2 in methylated control (Hela), HpaII enzyme cut both unmethylated and methylated alleles, Figure 3.2(C). Even though after the author found that Daudi cell line DNA might not a good representative unmethylated control due previous reported partial SHP1P2 methylation of this cell line. The experiment

was not repeated in control unmethylated WBC sample. The HpaII methylation-sensitive restriction enzyme was not the reliable method to detect SHP1P2 methylation in limited amount of DNA. This led to false-negative results of SHP1P2 methylation assessment.

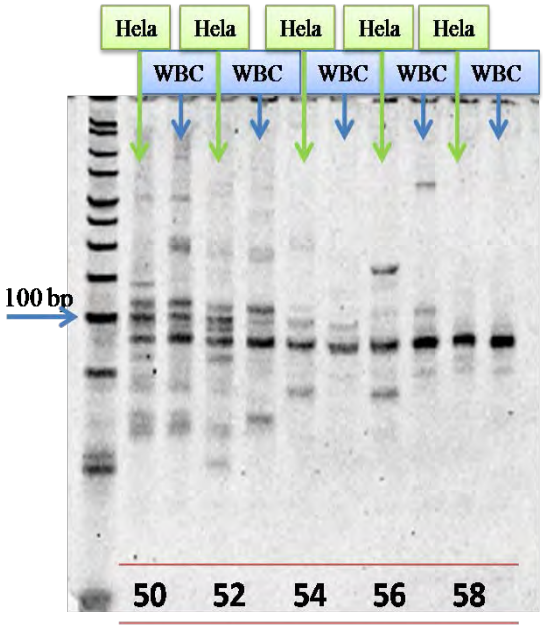


Figure 3.1 Gel electrophoresis of PCR product from ARMS primer set in control methylated sample (Hela) and control unmethylated sample (WBC), the number at the bottom represented the diverse annealing temperature of PCR reaction

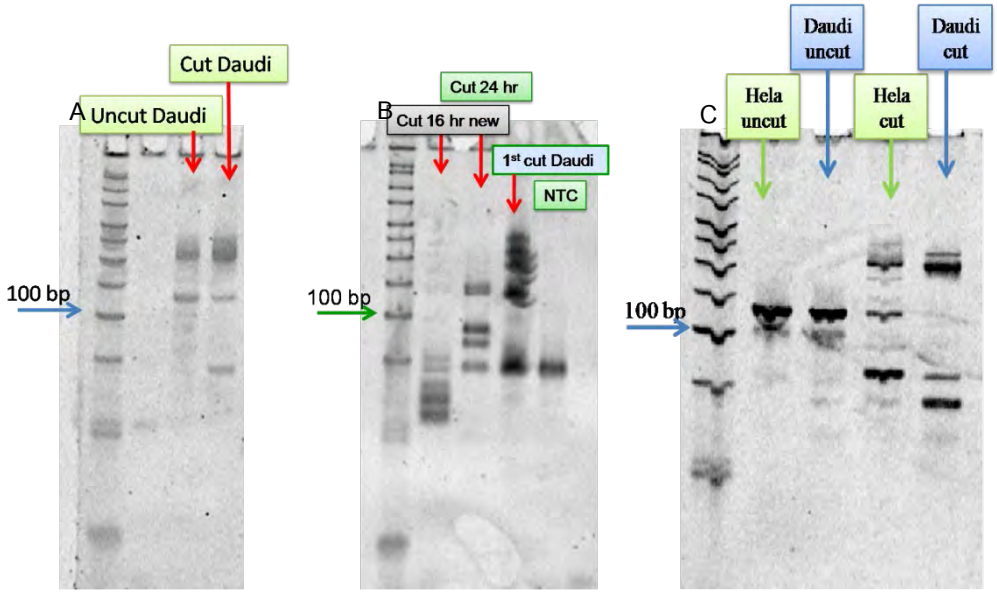


Figure 3.2 Standard treated HpaII methylation sensitive restriction enzyme-mediated PCR (A), modified protocol (B) and tested sample (C); target PCR product was 114-bp amplicon

3.3. DETECTION OF SHP-1 PROMOTER 2 METHYLATION BY CONVENTIONAL MSP-PCR

The 174-bp and 162-bp for methylated and unmethylated PCR amplicon respectively were visualized by gel electrophoresis on denaturing polyacrylamide gel, Figure 3.3. The PCR reaction of various known concentration of mixing control methylated and unmethylated was done. The SHP1P2 methylation index (MI) was calculated as a ratio of the intensity of the methylated amplicon divided by the sum of the methylated and unmethylated amplicon intensity measured by a phosphoimager using the Image Quant Software which shown linear correlation of the assay (= 0.78).

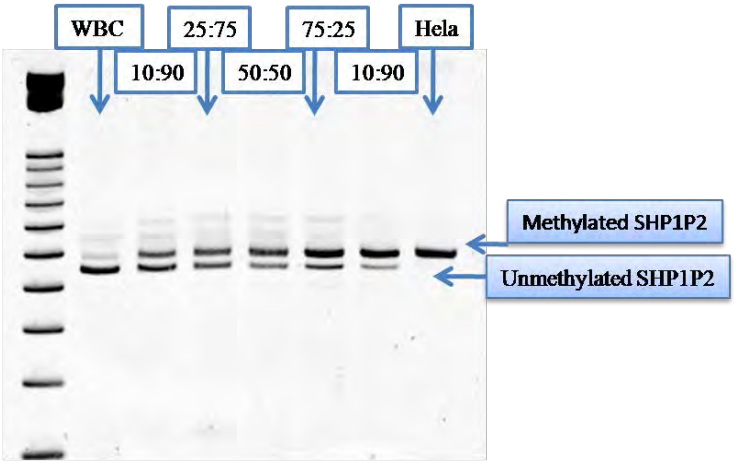


Figure 3.3 Gel electrophoresis of PCR product from MSP primer set in various concentration mixing control methylated sample (Hela) and control unmethylated sample (WBC)

3.4. QUANTITATIVE ANALYSIS OF SHP-1 PROMOTER 2 METHYLATION BY REAL-TIME PCR

Methylight assay in this study use the distinct methylated and unmethylation probe to discriminate between those proportions. The PCR product of methylated and unmethylated sequences was not separately visualized by gel electrophoresis. The detector channel from real-time PCR machine and signal from established standard curve were shown (figure 3.4). The lowest amount of bisulfite-treated DNA which could be amplified by this technique was evaluated. Using 10-fold dilution, 10 picograms of bisulfite-treated DNA was the lowest amount that could be detected by SHP1P2 methylight assay. Of these methylated SHP1P2 can be detected reliably in the presence of 100 fold excess of unmethylated alleles which is the lowest scale of sensitivity testing in known mixing sample.

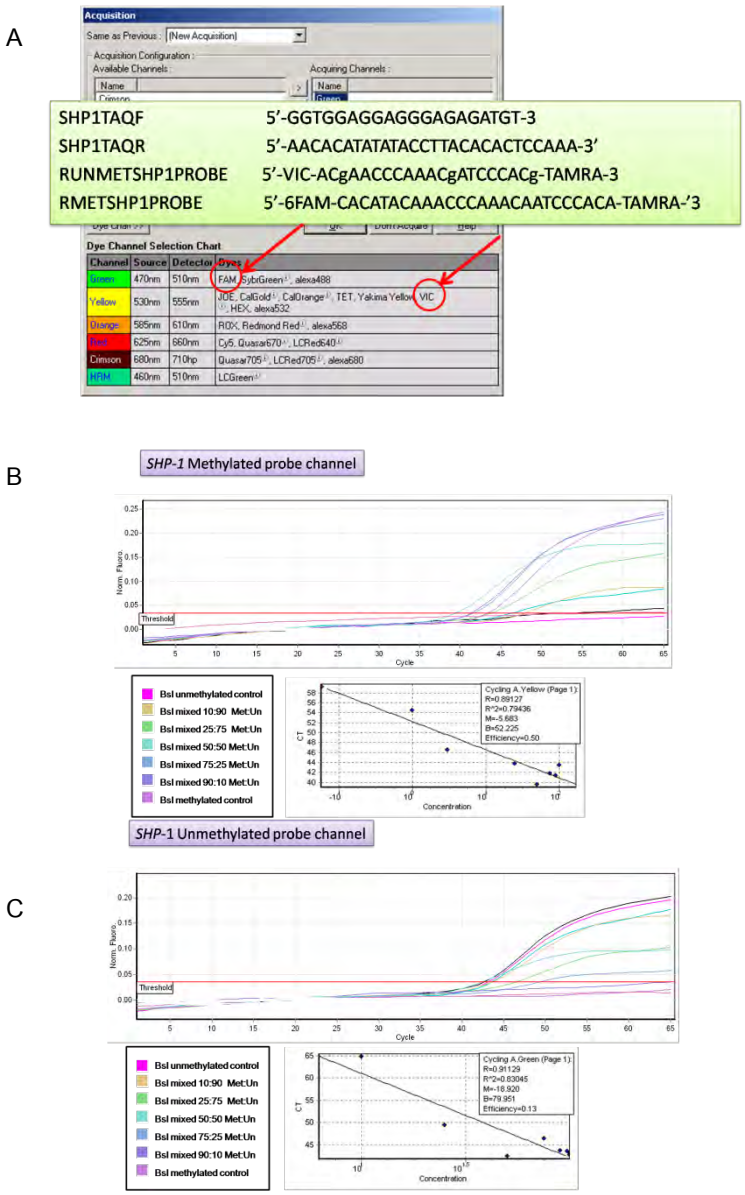


Figure 3.4 Real-time PCR channel of fluorescence detector (A), signal of standard curve detection according to channel; methylated (B) and unmethylated (C)

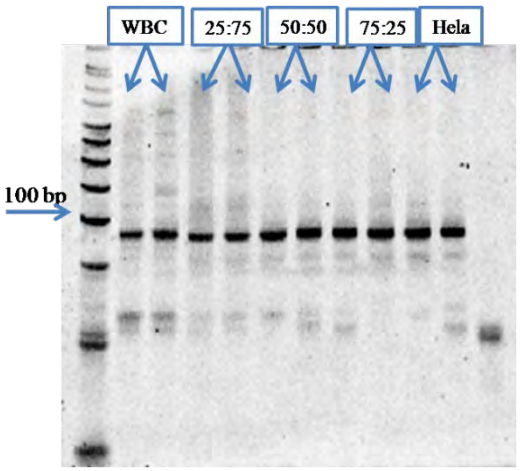


Figure 3.5 Gel electrophoresis of target 83-bp PCR product by methylight assay

The absolute quantitative version of quantitative assessment of methylated alleles (AQAMA) by real time PCR with specific dual hybridization probe for methylated and unmethylated sequences was compared with MSP-PCR method which previous reported the reliability validated with the bisulfite sequencing. The testing focused on limited amount of template condition by using SHP1P2 Methylation index from those two methods.

3.5. RELIABLE MEASUREMENT OF SHP-1 PROMOTER 2 METHYLATION BY QUANTITATIVE REAL-TIME PCR

To validate the methylight assay, the measurement of SHP1P2 methylation index by establishing MSP-PCR technique with the quantitative real-time PCR on a set of known concentration of the SHP1P2 methylation mixing samples in blind fashion was compared. Both MSP-PCR and AQAMA demonstrated linear correlations to the known concentration of the methylation of DNA sequence. However, the dual-probe technique showed a near-perfect linearity with Pearson's correlation coefficient of = 0.99, whereas MSP-PCR had correlation coefficient of = 0.78 (Figure 3.6). Thus, it can be concluded that AQAMA serves as a reliable tool to determine precisely the level of methylation in a sample with limited amount of DNA.

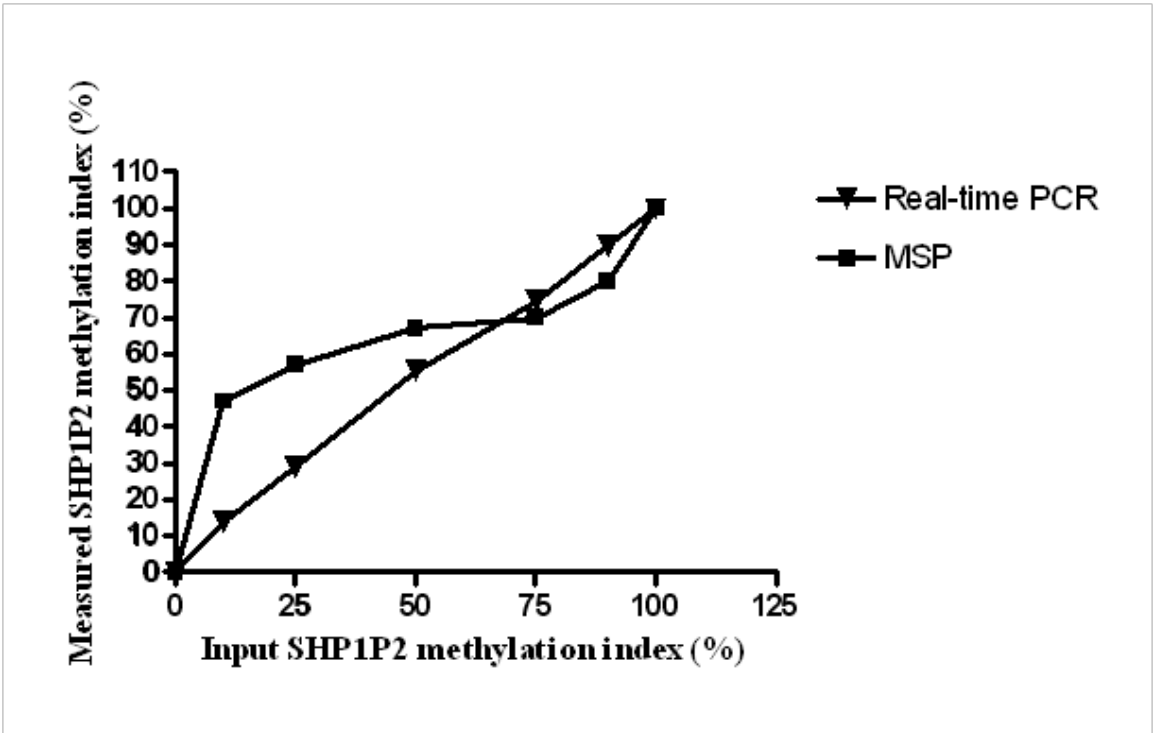


Figure 3.6 Standard curve analysis of AQAMA and MSP assays with known variable concentrations of DNA methylation input demonstrated linear correlation by Pearson's correlation coefficient analysis of both assays. Triangle represents data point from AQAMA and square represents MSP.

CHAPTER IV

RESULTS

PART II THE IMPLICATION OF SHP-1 PROMOTER 2 METHYLATION IN ADVANCED NON-SMALL CELL LUNG CANCER

4.1. USING SHP-1 PROMOTER 2 AS A PLASMA BIOMARKER IN NON-SMALL CELL LUNG CANCER

The SHP1P2 methylation was readily detectable in 35 of 38 plasma samples of advanced NSCLC, but majority undetectable in 33 of 52 plasma samples of healthy volunteer (Table 4.1). The analysis according to both absolute level of SHP1P2 methylation status and was SHP1P2 methylation index done.

4.1.1. SHP-1 PROMOTER 2 METHYLATION STATUS

The median absolute level of plasma SHP1P2 methylation status in advanced NSCLC was 0.77 ng ml^{-1} (range 0-26.5139 ng ml^{-1}) which was significantly higher and boarder range from healthy volunteer controls (range 0-0.2197 ng ml^{-1}, $p < 0.001$) (Figure 4.1(A)). The significant different of SHP1P2 methylation status between advanced NSCLC and healthy volunteer was found in all demographic parameter whether sex (male vs female), age (< 60 vs $\geq$ 60 yr) (Table 4.2). Among healthy volunteer control the plasma SHP1P2 methylation status was not significantly correlated either sex (Figure 4.2(A)) or age (Figure 4.3 (A)).

The area under the ROC curve between advanced NSCLC from sex/age match healthy volunteer was a value of 0.93 for SHP1P2 methylation status suggesting a strong discrimination power of the molecular assay to discriminate those 2 conditions (95% confidence interval was 0.86-0.99) (Figure 4.4). The level of SHP1P2 methylation status at cutoff level 0.0359 ng ml^{-1} was the best level of discriminate with sensitivity 89.5% and specificity 90.4% (Table 4.3).

Table 4.1 Detection rate of any level of SHP1P2 methylation in advanced NSCLC and sex/age match healthy volunteer

Group	Healthy controls	Advanced NSCLC	p-value
SHP1P2 methylation any level	20	35	$p<0.001$
No aberrant detection	33	3	
Total	52	38	

Table 4.2 Demographic data and median SHP1P2 methylation status and SHP1P2 methylation index of advanced NSCLC plasma and sex/age match healthy volunteer

Parameter	No of NSCLC	Median SHP1P2 methylation status (ng ml^{-1})	Median SHP1P2 methylation index (%)	No of control	Median SHP1P2 methylation status (ng ml^{-1})	Median SHP1P2 methylation index (%)	*p-values*
Age							
< 60 yr	21	0.8417 (0-4.5257)	16.8 (0-62.1)	27	0 (0-0.1156)	0 (0-9.7)	*<0.001*
≥ 60 yr	17	0.7084 (0-26.5139)	17.0 (0-62.6)	25	0 (0-0.2197)	0 (0-2.4)	*<0.001*
Sex							
Male	20	0.9340 (0.0005-4.5257)	20.3 (0-62.1)	23	0 (0-0.1156)	0 (0-9.7)	*<0.001*
Female	18	0.7084 (0-26.5139)	16.9 (0-62.6)	29	0 (0-0.2197)	0 (0-7.5)	*<0.001*
Smoking status							
Smoking	19	1.1596 (0.0005-6.6845)	22.2 (0-62.1)	0	N/A	N/A	
Non-smoking	19	0.6462 (0-26.5139)	16.8 (0-62.6)	52	0 (0-0.2197)	0 (0-9.7)	*<0.001*

4.1.2. SHP-1 PROMOTER 2 METHYLATION INDEX

The median plasma SHP1P2 index in advanced NSCLC was 17% (range 0-62.6) whereas SHP1P2 index in healthy volunteer was 0% (range 0-9.7) (p <0.001) (Figure 4.1). The significant difference was found in all demographic data correlated with afore-mentioned absolute level of SHP1P2 methylation status (Table 4.2). No correlation between SHP1P2 methylation index and age or sex in healthy volunteer group (Figure 4.2(B) and 4.3(B)). Using AUC-ROC curve also shown strong

discriminative power of SHP1P2 methylation index, AUC-ROC area was 0.92 (95% confidence interval 0.85-0.98) (Figure 4.4(B)). The SHP1P2 methylation index at 1.82% had the best sensitivity and specificity to detect advanced NSCLC of 89% and 90% respectively (Table 4.4).

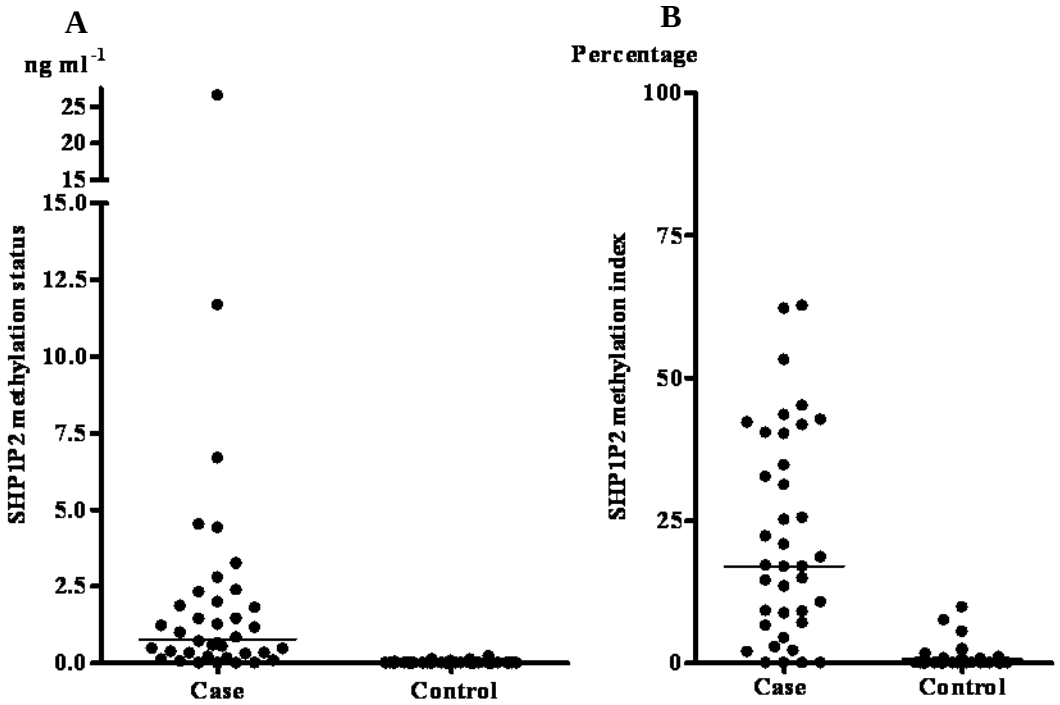


Figure 4.1 Distribution of plasma SHP1P2 methylation status (A) and methylation index (B) of advanced NSCLC and sex/age match healthy volunteer

Both parameters shown statistical significant different between advanced NSCLC and healthy volunteer. There showed the diagnostic potential of both absolute SHP1P2 methylation level and SHP1P2 index. This is pivotal data prove the concept of using SHP1P2 as plasma molecular marker in NSCLC. Nevertheless the author thought that the absolute among of SHP1P2 methylation might be better than SHP1P2 index to represent the amount of tumor-derived nucleic acid. Then sub sequential analysis to prove the prognostic potential of SHP1P2 was done comparative both parameters.

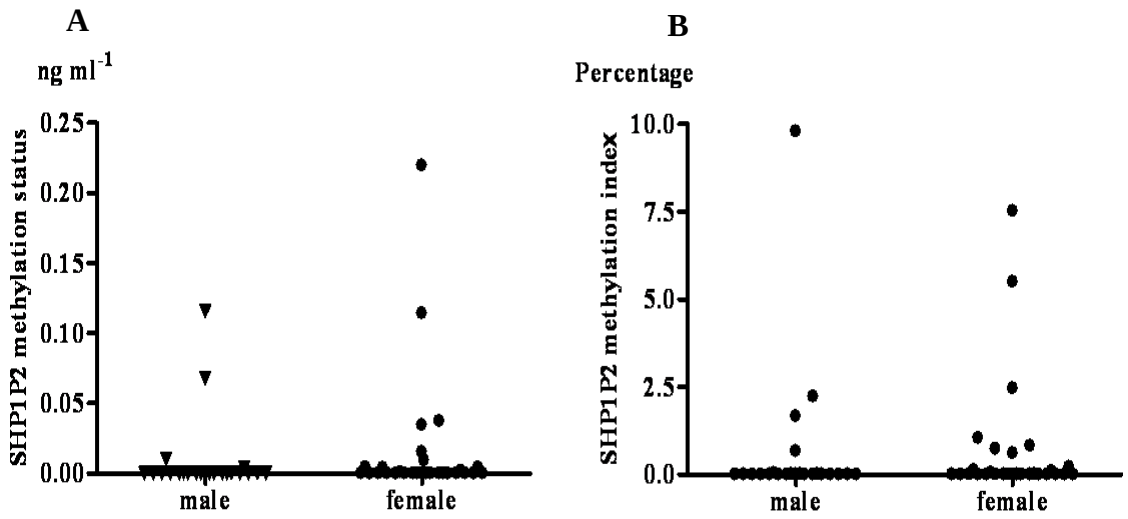


Figure 4.2 Distribution of plasma SHP1P2 methylation status (A) and methyaltion index (B) according to sex of healthy volunteer

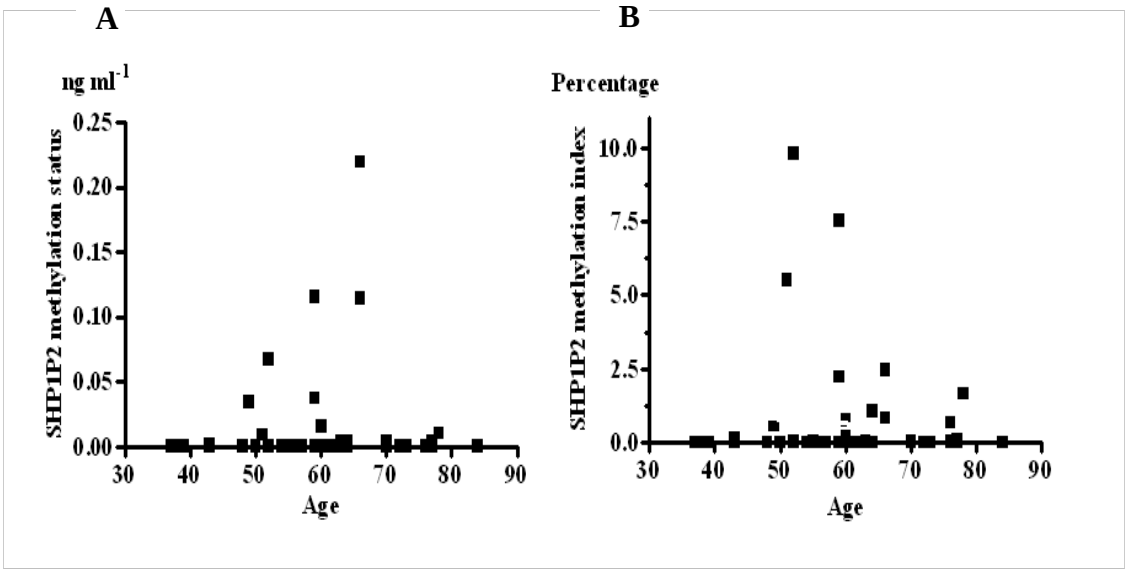


Figure 4.3 Distribution of SHP1P2 methylation status (A) and methylation index (B) according to age in plasma of healthy volunteer

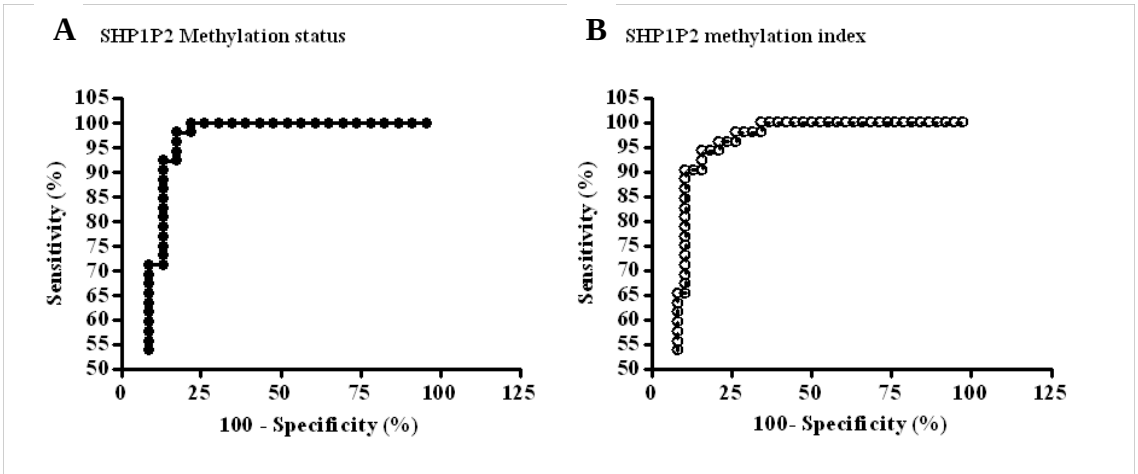


Figure 4.4 The area under the receiver operative characteristic curve of plasma SHP1P2 methylation status (A) and methylation index (B) to discriminate advanced NSCLC from healthy volunteer

Table 4.3 Difference absolute level of SHP1P 2 methylation status and power of discrimination advanced NSCLC from sex/age match healthy volunteer

Plasma SHP1P2 methylation cut-off (pg ml-1)	Sensitivity	Specificity
0.4	92.1%	71.2%
12.8	89.5%	86.5%
35.9	89.5%	90.4%
99.2	84.2%	94.2%
114.8	84.2%	96.2%

Table 4.4 difference level of SHP1P 2 methylation index and power of discrimination advanced NSCLC from sex/age match healthy volunteer

Plasma SHP1P2 methylation index (%)	Sensitivity (%)	Specificity (%)
0.93	89.5	86.5
1.35	89.5	88.5
1.82	89.5	90.4
2.06	86.8	90.4
2.18	84.2	90.4

4.2. PLASMA SHP-1 PROMOTER 2 METHYLATION AND ROLE OF PREDICTOR OF SURVIVAL IN ADVANCED NON-SMALL CELL LUNG CANCER

4.2.1. PATIENT CHARACTERISTICS

The enrolled study participants of advanced NSCLC were increased up to total 58 patients and enrolled in module 2. Among this population the median age was 59, (range 37-83). Sixty-seven percent of patients were male. The majority of NSCLC pathological type in this study was adenocarcinoma cell type (76%), the remaining cell types were squamous cell carcinoma 10.3%, large cell undifferentiated carcinoma 3.4% and NSCLC NOS 8.6%. All of the patients started their first-line of chemotherapy treatment for metastasis or relapsed disease. Doublet chemotherapy was use in 91% of the patient, paclitaxel and carboplatin was the common used regimen for 55% while gemcitabine and carboplatin was the second used regimen for 36%. Eastern Cooperative Oncology Group (ECOG) performance status 0-1 in this group was 91.4% (Table 4.4). Five patients dropped out of the study before the first follow-up visit and no response of treatment was assessed. Among this, three patients refused further treatment. Two patients died, one owing to severe anaphylactic reaction from chemotherapy and one owing to progression of disease. The overall clinical benefit rate by provided physician was 79.4%, composed of partial response 32.8% and stable disease 46.6%. At the end of this follow-up period (31 Dec 2009) 46.6% of the patients were still alive at the time of analysis while 10.7% of the patients had no disease progression. Median overall survival was 9.9 months (95% confidence interval 5.8-14.0). Median progression-free survival was 3.5 months (95% confidence interval 2.3-4.7 month). Median follow-up time in this study was 7.2 month (range 0.7-15.0 month). The overall treatment results were consistent with previous reports using the doublet chemotherapy treatment [79, 80].

Table 4.5 Demographic data of total study participants advanced NSCLC

Parameter	No of patient	%	Parameter	No of patient	%
Age			**Chemotherapy**		
< 60 yr	**30**	**52**	**Paclitaxel/carboplatin**	**32**	**55**
≥ 60 yr	**28**	**48**	**Gemcitabine/carboplatin**	**21**	**36**
Sex			**Carboplatin**	**5**	**9**
Male	**39**	**67**	**Pathological type**		
Female	**19**	**33**	**Adenocarcinoma**	**45**	**76**
Smoking status			**Squamous cell CA**	**6**	**11**
Smoking	**33**	**57**	**Large cell CA**	**2**	**4**
Non-smoking	**25**	**43**	**NSCLC NOS**	**5**	**9**
ECOG status			**Total regimen Rx**		
ECOG = 0	**6**	**10**	**< 2 regimen**	**35**	**60**
ECOG = 1	**47**	**81**	**≥ 2 regimen**	**23**	**40**
ECOG = 2	**5**	**9**	**All**	**58**	**100**

4.2.2. SHP-1 PROMOTER 2 METHYLATION OF NON-SMALL CELL LUNG CANCER STUDY PARTICIPANTS

4.2.2.1. ANALYSIS ACCORDING TO SHP-1 PROMOTER 2 METHYLATION STATUS

Baseline plasma SHP1P2 methylation status was obtained from 58 NSCLC patients. No SHP1P2 methylation was found in four plasma samples. The result consisted with the earlier-mentioned analysis in NSCLC patients versus healthy controls. The level of SHP1P2 methylation status was relatively high with a median of 0.6854 ng ml^{-1} (range: 0–28.3500 ng ml^{-1}). The level of SHP1P2 methylation status had no significant correlation to all demographic factors, including sex, age, smoking status, histology, chemotherapy regimen, and performance. The author analyzed level of SHP1P2 methylation status according the different various clinicopathological grouping such as sex, age (≥ or < 60 years), smoking status (yes or no), pathological cell type, regimen of first-line chemotherapy treatment, Eastern

Cooperative Oncology Group (ECOG) performance status (Table 4.5 and 4.6). Despite extreme plasma SHP1P2 methylation status in some categorical demographic data, there was no statistical significant difference between each parameter ($p > 0.01$) (Table 4.8 and 4.9).

4.2.2.2. ANALYSIS ACCORDING TO SHP-1 PROMOTER 2 METHYLATION INDEX

The analysis of SHP1P2 methylation index according to demographic data was done in the same manner of SHP1P2 methylation status. Median SHP1P2 index was 14.4% (0-71.5). No significant difference in subgroup of each demographic data including to sex, age, pathological cell type, ECOG performance status, TNM staging, site of metastasis, type of first-line chemotherapy treatment and response of treatment according to SHP1P2 methylation index (Table 4.6 and 4.7) (Table 4.10 and 4.11)

4.2.3. STRATIFICATION ACCORDING TO LEVEL OF SHP-1 PROMOTER 2 METHYLATION IN NON-SMALL CELL LUNG CANCER

To evaluate whether SHP1P2 methylation could be predictor of survival, the stratification according to baseline level of SHP1P2 methylation was done. To detect unfavorable prognosis group, defined as the patient who had an overall survival (OS) less than the median OS in this study, the ROC curve analysis between the baseline level of SHP1P2 methylation and OS outcome was carried out. For absolute level of SHP1P2 methylation status, the cutoff level at 0.07ng ml^{-1} was the most appropriate level to determine the survival of NSCLC patients in this study with a sensitivity of 68% and a specificity of 70%.

In the same manner, for SHP1P2 methylation index, the cut off value 11% was the best value and could be detected unfavorable prognosis with the sensitivity 71% and specificity 54%. The area under the AUC-ROC was 0.67 (95% confidence interval 0.54-0.81)

Table 4.6 Plasma SHP1P2 methylation status and SHP1P2 methylation index according to demographic data part 1

Parameter	No	Median SHP1P2 methylation status (ng ml^{-1})	Median SHP1P2 methylation index (%)
Age			
< 60 yr	**30**	**0.6547 (0-28.35)**	**15.8 (0-71.5)**
≥ 60 yr	**28**	**0.6854 (0-26.51)**	**13.9 (0-63.5)**
Sex			
Male	**39**	**0.7085 (0-28.35)**	**14.4 (0.01-71.5)**
Female	**19**	**0.5751 (0-26.51)**	**13.4 (0-62.6)**
Smoking status			
Smoking	**33**	**0.7888 (0-13.54)**	**18.5 (0.01-63.5)**
Non-smoking	**25**	**0.5751 (0-28.35)**	**13.4 (0-71.5)**
ECOG status			
ECOG = 0	**6**	**0.7486 (0.46-1.82)**	**17.0 (2.7-45.1)**
ECOG = 1	**47**	**0.6463 (0-26.51)**	**14.3 (0-63.5)**
ECOG = 2	**5**	**1.9887 (0.16-28.35)**	**43.7 (9.0-71.5)**
Chemotherapy			
Paclitaxel/carboplatin	**32**	**0.7200 (0-28.35)**	**14.6 (0-71.5)**
Gemcitabine/carboplatin	**21**	**0.3935 (0-11.68)**	**11.5 (0-62.6)**
Carboplatin	**5**	**1.9887 (0.16-13.54)**	**34.7 (10.6-63.5)**
Pathological type			
Adenocarcinoma	**45**	**0.5751 (0-28.35)**	**12.3 (0-71.5)**
Squamous cell CA	**6**	**1.2126 (0.06-6.68)**	**41.4 (1.5-49.3)**
Large cell CA	**2**	**1.7195 (0.65-2.79)**	**22.8 (14.4-31.2)**
NSCLC NOS	**5**	**0.7888 (0.31-1.87)**	**19.6 (6.5-32.6)**
Total regimen Rx			
< 2 regimen	**37**	**0.7085 (0-28.35)**	**14.3 (0-71.5)**
≥ 2 regimen	**21**	**0.5751 (0-4.53)**	**22.1 (0.01-45.1)**

Table 4.7 Plasma SHP1P2 methylation status and SHP1P2 methylation index according to demographic data part 2

Parameter	No	Median SHP1P2 methylation status (ng ml^{-1})	Median SHP1P2 methylation index (%)
T stage			
T 1	**5**	**0.3935 (0.32-1.22)**	**12.3 (6.5-28.9)**
T 2	**25**	**0.5426 (0-28.35)**	**10.6 (0.01-71.5)**
T 3	**7**	**1.2656 (0.58-6.68)**	**31.2 (2.7-42.6)**
T 4	**21**	**0.9926 (0-26.51)**	**18.5 (0-53.1)**
N stage			
N 0	**16**	**1.3032 (0.05-26.51)**	**22.1 (1.5-62.6)**
N 1	**3**	**0.1986 (0.09-1.45)**	**10.6 (9.1-17.0)**
N 2	**25**	**0.5426 (0-6.68)**	**13.4 (0-49.3)**
N 3	**14**	**0.8802 (0-28.35)**	**13.9 (0.01-71.5)**
M stage			
M 0	**6**	**0.9058 (0.32-6.68)**	**30.1 (2.7-49.6)**
M 1	**52**	**0.6120 (0-28.35)**	**13.9 (0-71.5)**
No of distant metastasis			
0 site	**5**	**0.9925 (0.71-6.68)**	**41.7 (2.7-49.3)**
1 site	**23**	**0.4746 (0-26.51)**	**9.05 (0-53.1)**
2 site	**24**	**0.5642 (0-13.54)**	**15.6 (0.01-63.5)**
3 site	**5**	**1.8158 (0.39-28.35)**	**40.3 (12.3-71.5)**
Response to Rx			
Partial response	**19**	**0.5533 (0-28.35)**	**14.3 (0-71.5)**
Stable disease	**27**	**0.7315 (0-26.51)**	**18.5 (0-63.5)**
Progression disease	**7**	**1.4469 (0.3-11.68)**	**16.8 (3.0-62.6)**
Not available	**5**	**0.4647 (0-1.99)**	**2.7 (0-34.7)**

Table 4.8 Demographic data of advanced NSCLC according to SHP1P2 methylation status stratification part 1

Parameter	No	SHP1P2 methylation status < 0.7 ng ml^{-1}	SHP1P2 methylation status ≥ 0.7 ng ml^{-1}	*P-value*
Age				
< 60 yr	**30**	**15**	**15**	***1.00***
≥ 60 yr	**28**	**14**	**14**	
Sex				
Male	**39**	**19**	**20**	***0.78***
Female	**19**	**10**	**9**	
Smoking status				
Smoking	**33**	**15**	**18**	***0.42***
Non-smoking	**25**	**14**	**11**	
ECOG status				
ECOG = 0	**6**	**2**	**4**	***0.58***
ECOG = 1	**47**	**25**	**22**	
ECOG = 2	**5**	**2**	**3**	
Chemotherapy				
Paclitaxel/carboplatin	**32**	**15**	**17**	***0.68***
Gemcitabine/carboplatin	**21**	**12**	**9**	
Carboplatin	**5**	**2**	**3**	
Pathological type				
Adenocarcinoma	**45**	**25**	**20**	***0.33***
Squamous cell CA	**6**	**1**	**5**	
Large cell CA	**2**	**1**	**1**	
NSCLC NOS	**5**	**2**	**3**	
Total regimen Rx				
< 2 regimen	**37**	**18**	**19**	***0.78***
≥ 2 regimen	**21**	**11**	**10**	

Table 4.9 Demographic data of advanced NSCLC according to SHP1P2 methylation status stratification part 2

Parameter	No	SHP1P2 methylation status < 0.7 ng ml^{-1}	SHP1P2 methylation status ≥ 0.7 ng ml^{-1}	*P-value*
T stage				
T 1	**5**	**3**	**2**	***0.07***
T 2	**25**	**17**	**8**	
T 3	**7**	**2**	**5**	
T 4	**21**	**7**	**14**	
N stage				
N 0	**16**	**6**	**10**	***0.21***
N 1	**3**	**2**	**1**	
N 2	**25**	**16**	**9**	
N 3	**14**	**5**	**9**	
M stage				
M 0	**6**	**1**	**5**	***0.19***
M 1	**52**	**28**	**24**	
Distant metastasis				
Bone	**15**	**6**	**9**	***0.36***
Lung	**33**	**19**	**14**	***0.18***
Liver	**6**	**4**	**2**	***0.67***
Lymph node	**5**	**1**	**4**	***0.35***
Brain	**13**	**9**	**4**	***0.11***
Pleura	**6**	**1**	**5**	***0.19***
Other	**9**	**4**	**5**	***1.0***
Response to Rx				
Partial response	**19**	**11**	**8**	***0.57***
Stable disease	**17**	**13**	**14**	
Progressive disease	**7**	**2**	**5**	
Not available	**5**	**3**	**2**	

Table 4.10 Demographic data of advanced NSCLC according to SHP1P2 methylation index stratification part 1

Parameter	No	SHP1P2 methylation Index < 11%	SHP1P2 methylation index ≥ 11%	*P-value*
Age				
< 60 yr	**30**	**13**	**17**	***0.75***
≥ 60 yr	**28**	**11**	**17**	
Sex				
Male	**39**	**16**	**23**	***0.93***
Female	**19**	**8**	**11**	
Smoking status				
Smoking	**33**	**14**	**19**	***0.85***
Non-smoking	**25**	**10**	**15**	
ECOG status				
ECOG = 0	**6**	**3**	**3**	***0.90***
ECOG = 1	**47**	**19**	**28**	
ECOG = 2	**5**	**2**	**3**	
Chemotherapy				
Paclitaxel/carboplatin	**32**	**13**	**19**	***0.52***
Gemcitabine/carboplatin	**21**	**10**	**11**	
Carboplatin	**5**	**1**	**4**	
Pathological type				
Adenocarcinoma	**45**	**21**	**24**	***0.32***
Squamous cell CA	**6**	**1**	**5**	
Large cell CA	**2**	**0**	**2**	
NSCLC NOS	**5**	**2**	**3**	
Total regimen Rx				
< 2 regimen	**37**	**16**	**21**	***0.70***
≥ 2 regimen	**21**	**8**	**13**	

Table 4.11 Demographic data of advanced NSCLC according to SHP1P2 methylation index stratification part 2

Parameter	No	SHP1P2 methylation index < 11%	SHP1P2 methylation index ≥ 11%	*P-value*
T stage				
T 1	**5**	**2**	**3**	***0.25***
T 2	**25**	**14**	**11**	
T 3	**7**	**2**	**5**	
T 4	**21**	**6**	**15**	
N stage				
N 0	**16**	**5**	**11**	***0.55***
N 1	**3**	**2**	**1**	
N 2	**25**	**12**	**13**	
N 3	**14**	**5**	**9**	
M stage				
M 0	**6**	**2**	**4**	***0.67***
M 1	**52**	**22**	**30**	
Distant metastasis				
Bone	**15**	**3**	**12**	***0.05***
Lung	**33**	**14**	**19**	***0.85***
Liver	**6**	**3**	**3**	***0.65***
Lymph node	**5**	**0**	**5**	***0.07***
Brain	**13**	**6**	**7**	***0.69***
Pleura	**6**	**1**	**5**	***0.19***
Other	**9**	**5**	**4**	***0.46***
Response to Rx				
Partial response	**19**	**6**	**13**	***0.22***
Stable disease	**17**	**12**	**15**	
Progressive disease	**7**	**2**	**5**	
Not available	**5**	**4**	**1**	

4.2.4. PLASMA SHP-1 PROMOTER 2 METHYLATION AS A PROGNOSTIC FACTOR OF SURVIVAL

Following the chosen cutoff level of 0.70 ng ml^{-1} for further analysis, the authors performed additional survival analyses to confirm the significance of absolute level of SHP1P2 methylation status as a potential biomarker. Patients who had a baseline plasma SHP1P2 methylation status $\geq$ 0.70 ng ml^{-1} had shorter progression-free survival (PFS) of 2.6 months (95% confidence interval 1.3–3.8) than those who had a plasma SHP1P2 methylation level of < 0.70 ng ml^{-1} with PFS of 5.2 months (95% confidence interval 2.7–7.7) (p = 0.009). Similarly, patients with a higher absolute level of SHP1P2 methylation status had an overall survival (OS) of 7.6 months (95% confidence interval 3.5–11.6) when compared with 12.6 months (95% confidence interval 10.7–14.4) for those who had lower level of SHP1P2 methylation status (p = 0.01) (Figure 4.6). Moreover, the univariate analysis for an independent prognostic factor for survival outcome was performed, including age, sex, smoking status, performance status, number of systemic treatment regimen, and SHP1P2 methylation status in advanced NSCLC patients. The result revealed that among the baseline pretreatment clinical data SHP1P2 methylation status was the only significant prognostic factor for both PFS and OS with p = 0.01 and 0.01, respectively (Table 4.12). Under the multivariate analysis model, the absolute level of SHP1P2 methylation status was the important prognostic factor with the hazard ratio for disease recurrence and death by plasma SHP1P2 methylation status of $\geq$ 0.70 ng ml^{-1} equal to 1.482 (95% confidence interval 1.09 –2.00) and 2.710 (95% confidence interval 1.20 –6.58), respectively. Furthermore the number of systemic treatment regimen also affected with survival outcome. The patient who received only first-line systemic treatment had significant worse overall survival outcome than patient who received more than first-line systemic treatment, by multivariate analysis hazard ratio of death equal to 2.80 (95% confidence interval 1.19 - 6.58) *(p = 0.01*).These results indicate that the pretreatment level of SHP1P2 methylation in plasma is an important prognostic factor in advanced NSCLC.

Using cutoff value of 11% SHP1P2 methylation index to classify favorable and unfavorable prognosis grouping, median OS in favorable prognosis which SHP1P2 methylation index less than 11% was 12.6 months (95% confidence interval

6.2-19.0) while unfavorable prognosis was 8.2 months (95% confidence interval 3.7-12.7). No statistical significant difference was found, p = 0.10. Consistent with progression-free survival analysis which was 4.3 months in favorable group and 3.5 months in unfavorable prognosis group, p = 0.15 (Figure 4.6). The result of SHP1P2 methylation index could not be used as the biomarker to predict survival outcome in advanced NSCLC. No further analysis according to SHP1P2 methylation index was done.

4.2.5. ROLE OF PLASMA SHP-1 PROMOTER 2 METHYLATION AND THE RESPONSE OF TREATMENT

Conventionally, response to treatment of NSCLC with chemotherapy may require a few cycles of treatment prior to response evaluation. To further explore the potential of applying SHP1P2 methylation status as a potential tool for early monitoring of the response to chemotherapy, the level of SHP1P2 methylation status in plasma was serially measured before second cycle of treatment. There were 42 follow-up blood samples available for this analysis. Response to chemotherapy was provided by the treating physicians according to their standard practice. The response of treatment was correlated with progression-free survival of the patient (Table 4.13). The authors found no significant correlation of the difference between follow-up and baseline level of plasma SHP1P2 methylation status and the response of chemotherapy treatment (Figure 4.7). Additional analyses showed that the decrement or increment of the SHP1P2 methylation also expressed no correlation to both PFS and OS outcomes with p = 0.133 and 0.995, respectively. The follow-up level of SHP1P2 methylation status could not be used as early monitoring treatment response of chemotherapy treatment in advanced NSCLC.

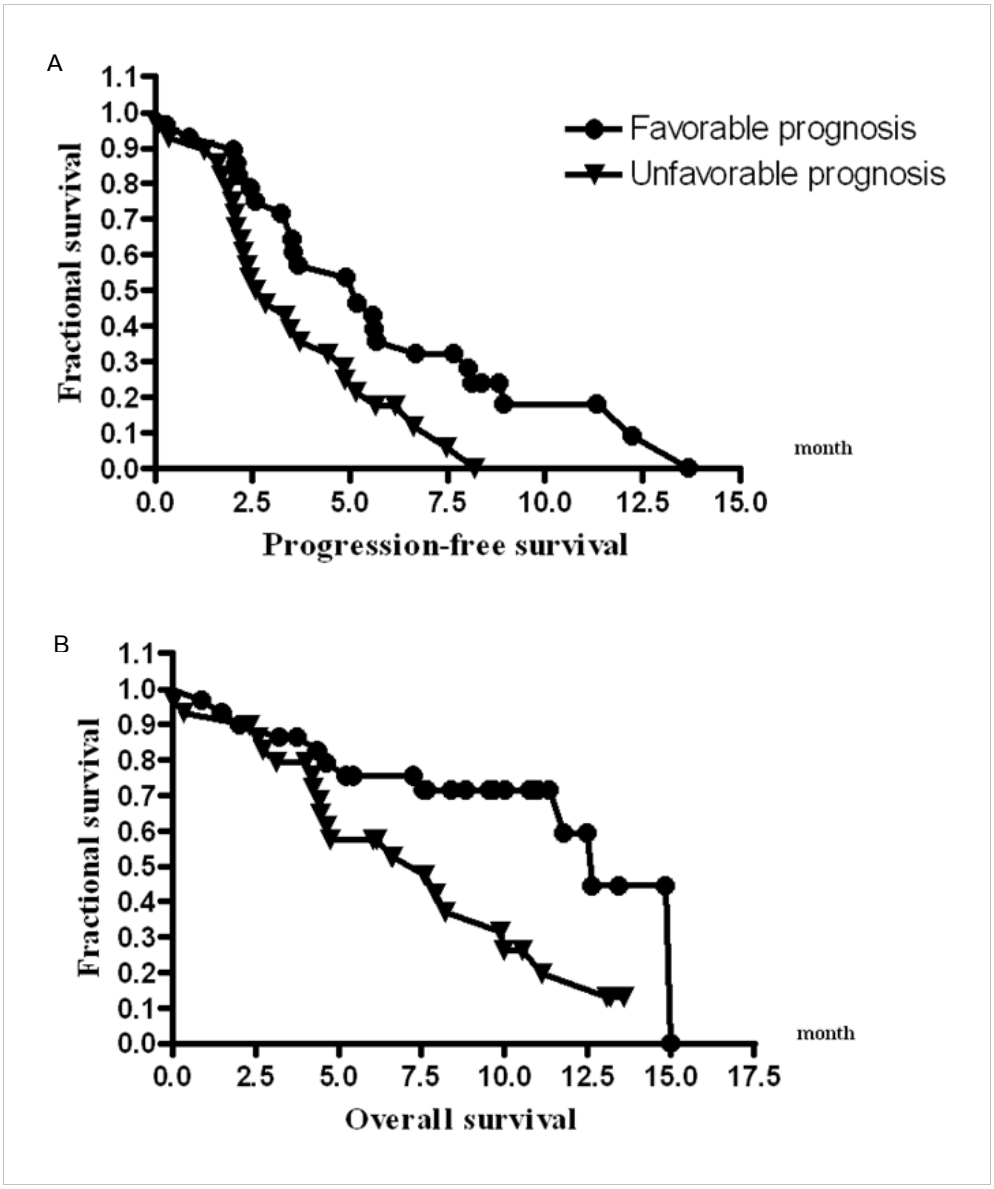


Figure 4.5 Plotted survival curve demonstrates progression-free survival (A) and overall survival (B) of advanced NSCLC patients according to plasma SHP1P2 methylation status

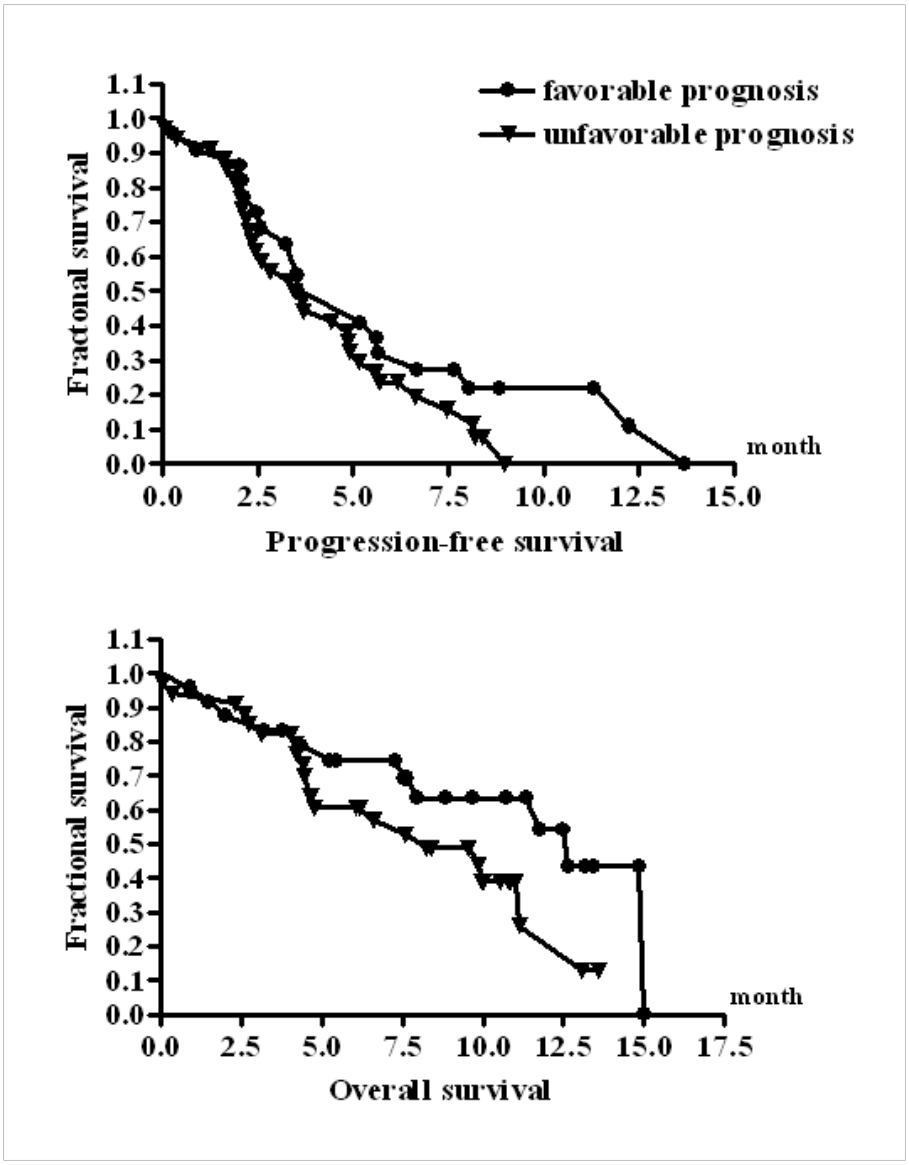


Figure 4.6 Plotted survival curve demonstrates progression-free survival (A) and overall survival (B) of advanced NSCLC patients according to plasma SHP1P2 methylation index

Table 4.12 Univariate and multivariate analysis of survival outcome using Cox's proportional hazard regression analysis including clinical parameters and plasma SHP1P2 methylation status

Factor	Univariate Hazard ratio of death (95% CI)	*p-value*	Multivariate Hazard ratio of death (95% CI)	*p-value*	Univariate Hazard ratio of recurrence (95% CI)	*p-value*	Multivariate Hazard ratio of recurrence (95% CI)	*p-value*
Sex male vs female	1.24 (0.56-2.72)	0.58	0.64 (0.19-2.12)	0.47	1.02 (0.75-1.39)	0.86	0.98 (0.63-1.52)	0.94
Age <60 vs ≥ 60 yr	1.12 (0.54-2.30)	0.75	1.38 (0.65-2.95)	0.39	1.03 (0.77-1.37)	0.82	1.00 (0.75-1.35)	0.96
Smoking status yes vs no	1.52 (0.72-3.21)	0.26	1.98 (0.57-6.84)	0.27	1.00 (0.75-1.33)	0.97	0.99 (0.64-1.52)	0.96
ECOG (0,1 vs 2)	0.79 (0.23-2.65)	0.70	0.34 (0.07-1.55)	0.16	0.72 (0.27-1.88)	0.50	0.67 (0.20-2.24)	0.52
Regimen Rx <2 vs ≥ 2	2.58 (1.13-5.91)	0.02	2.80 (1.19-6.58)	0.01*	ND[±]		ND[±]	
SHP1P2 methylation ≥ 0.7 vs < 0.7 ng ml^{-1}	2.36 (1.22-5.64)	0.01*	2.71 (1.20-6.07)	0.01*	1.47 (1.09-1.97)	0.01*	1.48 (1.09-2.00)	0.01*

ND = not determine

Table 4.13 The level of pretreatment and follow-up SHP1P2 methylation status according to response of first-line chemotherapy treatment

Response rate	Number	pretreatment SHP1P2 methylation	Follow-up SHP1P2 methylation	Median PFS (95% confidence interval)
Partial response	16	2.3238 ± 6.9524	2.2055 ± 5.7975	5.667 (3.837-7.496)
Stable disease	18	1.2212 ± 1.5389	3.7517 ± 8.3031	3.533 (3.120-3.947)
Progressive disease	6	1.7088 ±1.5969	0.9448 ± 1.2063	2.067 (1.947-2.187)
Total	40	1.7354 ± 4.4960	2.7122 ± 6.6520	4.900 (3.712-6.088)

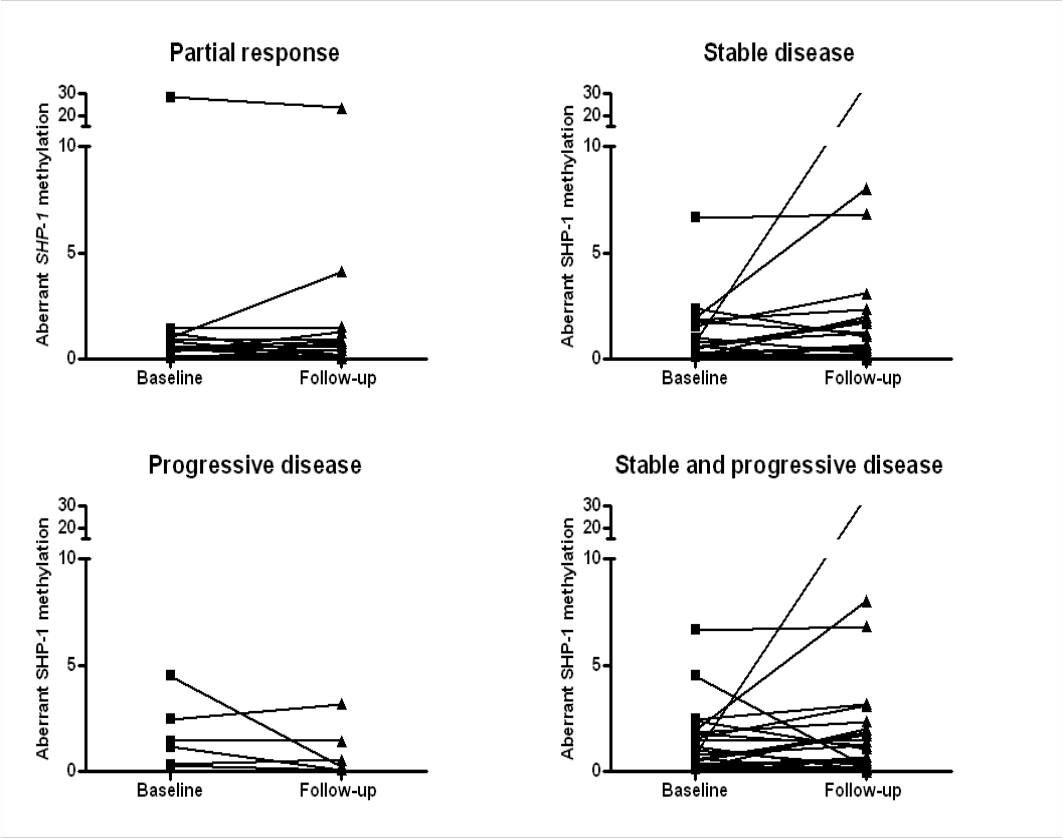


Figure 4.7 Alteration pretreatment and follow-up level of plasma SHP1P2 methylation status according to response of first-line chemotherapy treatment

CHAPTER V

RESULTS

Part III THE BIOLOGY OF CIRCULATING NUCLEIC ACID IN ADVANCED NON-SMALL CELL LUNG CANCER

5.1. THE CIRCULATIGN NUCLEIC ACID AND ITS BIOLOGY

5.1.1. THE CIRCULATING NUCLEIC ACID AND ITS COMPONENT

From first group enrollment of study participant advanced NSCLC and sex/age match healthy volunteer. The quantitative analysis of circulating DNA was obtained from 38 plasma samples of advanced NSCLC and 53 sex/age match plasma samples of healthy volunteer. The median circulating DNA in advanced NSCLC was 4.5 ng ml^{-1} (range 1.33-49.88). It was statistical significant difference from healthy volunteer. The median circulating DNA in latter group was 2.0 ng ml^{-1} (range 0.006-26.95, $p = 0.001$). No statistical significant difference was found in circulating DNA level according to demographic data. Among the component of circulating DNA, the sharp contrast fraction of biology between those groups was found. The SHP1P2 methylation index which represented tumor-derived nucleic acid was predominant in advanced NSCLC as mentioned in chapter IV (Table 5.1). In another way it could be concluded that the majority of healthy volunteer was all hematopoietic-derived nucleic acid proportion (range 90.3-100%). In advanced NSCLC there was also a trend of increasing absolute level of unmethylated SHP1P2 status (uSHP1P2) compare to healthy volunteer (*p-value = 0.026*). Median plasma uSHP1P2 level was 3.4 ng ml^{-1} (range 1.2-24.8) and 2.0 ng ml^{-1} (range 0.03-26.95) in advanced NSCLC and healthy volunteer respectively. (Figure 5.1)

5.1.2. THE CORRELATION OF UNMETHYLATED SHP-1 PROMOTER 2 AND LEUKOCYTE COUNTS

The majority of circulating DNA in healthy volunteer was uSHP1P2 consistent with previous Lui Y et al. study which demonstrate the majority of circulating nucleic acid derived from hematopoietic origin [12]. The author further explored potential source of hematopoietic-derived circulating nucleic acid. The postulate hypothesis was circulating hematopoietic cell such as white blood cell (WBC) and its component. Then the correlation of absolute amount of plasma uSHP1P2 level, total WBC count and its component was done in healthy volunteer group. There was neither correlation of uSHP1P2 and total WBC count nor WBC component, both neutrophil and lymphocyte (= 0.007, -0.03 and -0.01, p = 0.95, 0.81 and 0.94 respectively). Furthermore there was also neither correlation of uSHP1P2 and hemoglobin nor platelet component (= 0.05, -0.08, p = 0.67 and 0.53 respectively).

Table 5.1 Circulating DNA level and SHP1P2 methylation index of advanced NSCLC and sex/age match healthy volunteer

Parameter	Circulating DNA level (ng ml^{-1}) advanced NSCLC	SHP1P2 methylation index (%) advanced NSCLC	Circulating DNA level ($ngml^{-1}$) healthy volunteer	SHP1P2 methylation index (%) healthy volunteer
Age				
< 60 yr	**5.7695 ± 5.5027**	**16.8 (0-62.1)**	**4.8406 ± 6.6517**	**0 (0-9.7)**
≥ 60 yr	**10.0376 ± 12.1764**	**17.0 (0-62.6)**	**4.3951 ± 4.9848**	**0 (0-2.4)**
Sex				
Male	**7.4433 ± 6.9063**	**20.3 (0-62.1)**	**5.8225 ± 7.0784**	**0 (0-9.7)**
Female	**7.9408 ± 11.4798**	**16.9 (0-62.6)**	**3.6779 ± 4.5827**	**0 (0-7.5)**
Smoking status				
smoking	**7.9299 ± 7.2290**	**22.2 (0-62.1)**	**ND±**	**ND±**
Non-smoking	**7.4280 ± 11.0702**	**16.8 (0-62.6)**	**4.6264 ± 5.8566**	**0 (0-9.7)**
All	**7.6789 ± 9.2253**	**17.0 (0-62.6)**	**4.6264 ± 5.8566**	**0 (0-9.7)**

The analysis was done in the same manner in advanced NSCLC. There was no correlation of plasma uSHP1P2 and WBC count including its component (neutrophil and lymphocyte) in advanced NSCLC (= 0.21, 0.18 and 0.01, p = 0.23, 0.3 and 0.91 respectively).

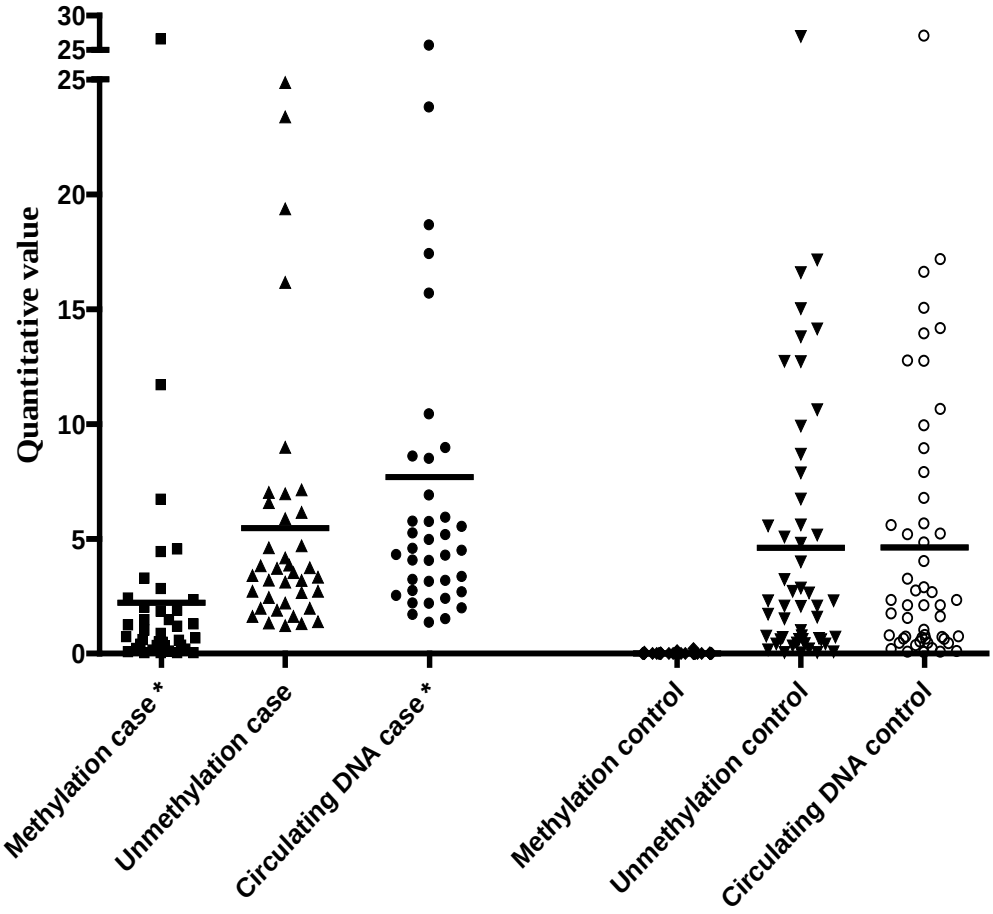


Figure 5.1 Plotted of absolute level of plasma mSHP1P2, uSHP1P2 and total circulating DNA level according to advanced NSCLC and healthy volunteer (* $p \leq 0.01$)

5.1.3. THE CORRELATION OF METHYLATED AND UNMETHYLATED SHP-1 PROMOTER 2 IN ADVANCED NON-SMALL CELL LUNG CANCER

The interaction of malignancy and immune system is counteracting process and modified by each other. The correlation between absolute amount of plasma

SHP1P2 methylation status (mSHP1P2) and uSHP1P2 level was an interesting issue to explore. The author found that there was statistical significant correlation between plasma uSHP1P2 and mSHP1P2 level in advanced NSCLC both pretreatment and follow-up before second cycle of chemotherapy treatment (= 0.22, p = 0.0002 and = 0.54, p = 0.0001 respectively) (Figure 5.2). Still now unclear mechanism of tumor-bearing host and inflammatory cell, however the correlation of tumor and inflammatory-derived nucleic acid did not impact by chemotherapy treatment.

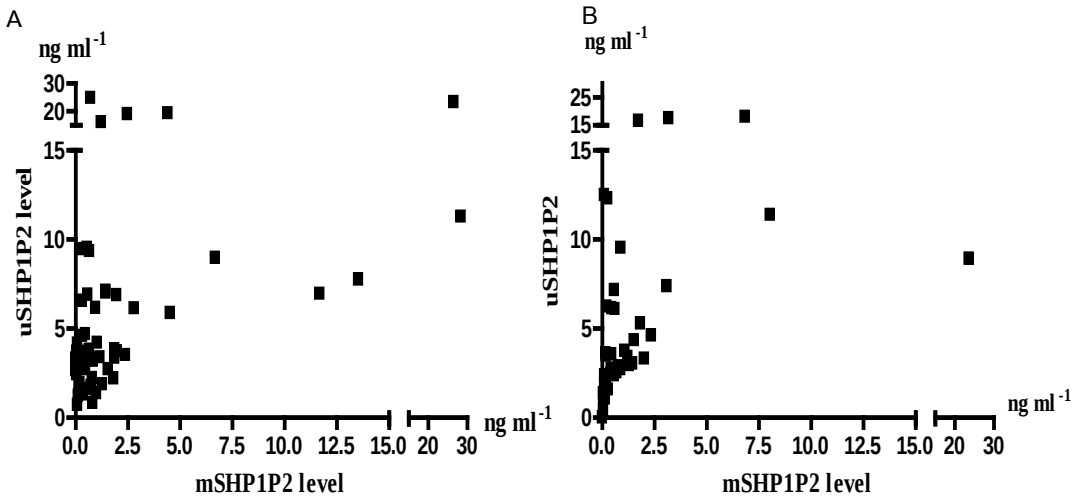


Figure 5.2 The correlation of plasma mSHP1P2 and uSHP1P2 level in advanced NSCLC pretreatment (A) and follow-up (B). Significant correlation of those parameters were found by Spearman's correlation

5.1.4. COMPARATIVE POWER OF DISCRIMINATION BETWEEN ADVANCED NON-SMALL CELL LUNG CANCER AND CONTROL

The author explored the potential valuable biomarker to discriminate advanced NSCLC from healthy volunteer, whether mSHP1P2, uSHP1P2 or circulating DNA level. Using the receiver operating characteristic curve operate in the same manner. Plasma mSHP1P2 was the most powerful biomarker to discriminate advanced NSCLC from healthy volunteer control. Area under the receiver operating characteristic (ROC) curve of mSHP1P2, circulating DNA level and uSHP1P2 were

0.93, 0.69 and 0.63 respectively (Figure 5.3). Consistent with result in chapter IV, role of SHP1P2 as a potential diagnostic marker is better than circulating DNA.

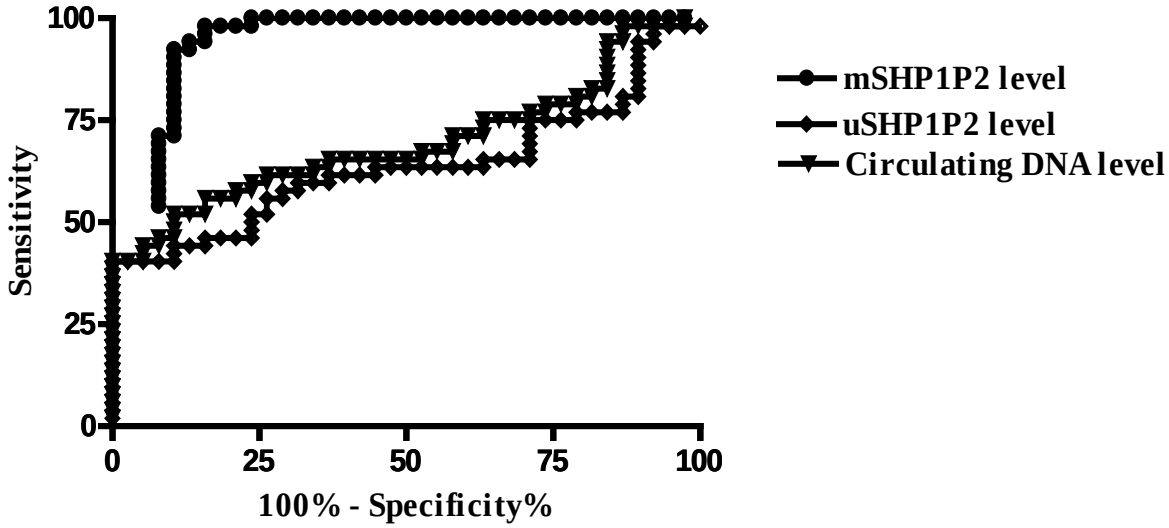


Figure 5.3 Area under the receiver operative characteristic curve (ROC) of mSHP1P2, uSHP1P2 and circulating DNA level to discriminate advanced NSCLC from healthy volunteer

5.1.5. EXPLORATORY ANALYSIS OF PLASMA SHP1P2 METHYLATION IN EARLY-STAGE NSCLC

To further expand our understanding of the role of plasma SHP1P2 methylation as a biomarker in NSCLC, an exploratory cohort of 20 resectable NSCLC cases (Table 5.2) was investigated. Consistent with the analyses in advanced NSCLC patients, the level of SHP1P2 methylation in stage I-III NSCLC patients was apparently higher than in healthy controls, with a median value of 1.24 ng ml^{-1} [0.35-10.48 ng ml^{-1}; p <0.001]. Interestingly, the total circulating DNA levels in the plasma measured in these early-stage NSCLC patients were not significantly different from those of the controls. The median circulating DNA levels were 1.88 ng ml^{-1} [0.37-17.54 ng ml^{-1}] in resectable NSCLC patients compared with 2.07 ng ml^{-1} [0.034-26.95 ng ml^{-1}] in the healthy controls (p = 0.36) (Figure 5.4). This result indicated that the level of circulating DNA was a less sensitive and specific biomarker than SHP1P2

methylation. Accordingly, only levels of SHP1P2 methylation can be employed to distinguish patients with early stages of NSCLC from healthy controls.

Table 5.2 Baseline characteristics of patients with resectable NSCLC

Characteristic	No of Patients (%)
NSCLC patients	**20**
Age, in years	
Median	**61 years**
Range	**45-83 years**
Sex	
Female	**4 (20)**
Male	**16 (80)**
Histology	
Adenocarcinoma	**14 (70)**
Squamous cell carcinoma	**4 (20)**
Large cell carcinoma	**1 (5)**
Other	**1 (5)**
Smoking status	
Smoker	**12 (60)**
Non-smoker	**8 (40)**
Staging UICC	
Stage I	**13 (65)**
Stage II	**-**
Stage III	**7 (35)**
Adjuvant chemotherapy	
Yes	**6 (30)**
No	**14 (70)**
Adjuvant radiation	
Yes	**4 (20)**
No	**16 (80)**

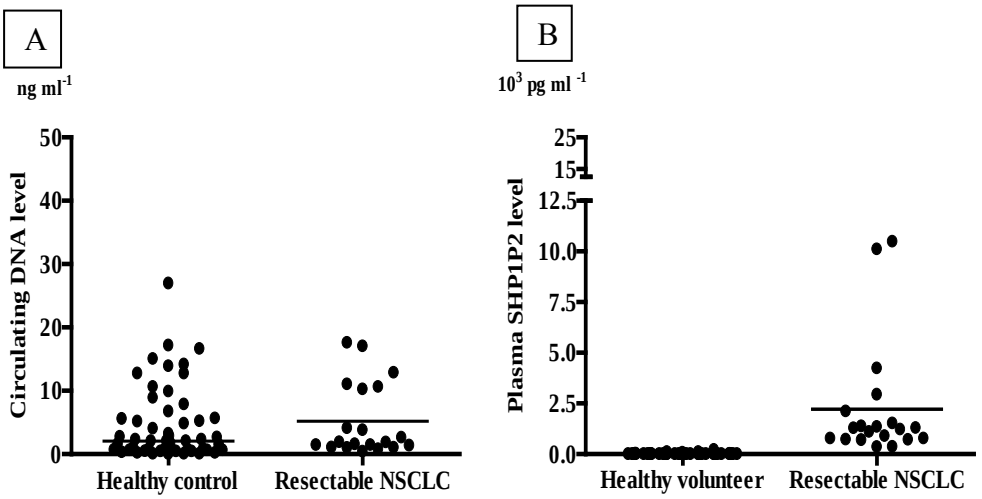


Figure 5.4 Plasma levels of SHP1P2 methylation were analyzed in 20 patients with resectable stages I-III NSCLC. The total quantity of circulating DNA (panel A) exhibited no significant difference between patients with early NSCLC and the healthy controls (p = 0.36). In contrast, the levels of SHP1P2 methylation (panel B) were higher and broader in patients with early stages of NSCLC compared with those of the healthy controls (p < 0.001).

5.2. THE IMPACT OF CIRCULATING DNA LEVEL IN ADVANCED NON-SMALL CELL LUNG CANCER, THE PREDICTOR OF SURVIVAL

5.2.1. CIRCULATING DNA LEVEL AND UNMETHYLATED SHP-1 PROMOTER 2

The clinicopathological data, circulating DNA level and plasma uSHP1P2 level of total 58 advanced NSCLC were analyzed for the impact to survival. The demographic data of patient was shown in chapter IV. The median circulating DNA level and plasma uSHP1P2 level in NSCLC were 4.3 ng ml^{-1} (range 0.8-49.8 ng ml^{-1}) and 3.6 ng ml^{-1} (range 0.7-24.8 ng ml^{-1}) respectively.

Among advanced NSCLC group, the author analyzed circulating DNA level and plasma uSHP1P2 according the each clinicopathological parameter such as sex (male or female), age ($\geq$ or < 60 years), smoking status (yes or no), pathological cell type, regimen of first-line chemotherapy treatment, Eastern Cooperative Oncology

Group (ECOG) performance status (0,1 vs 2). The circulating DNA level and plasma uSHP1P2 level were not statistical significant difference in each parameter. (Table 5.3)

5.2.2. STRATIFICATION ACCORDING TO CIRCULATING DNA AND UNMETHYLATED SHP-1 PROMOTER 2 LEVEL

To evaluate whether which molecular marker among circulating DNA level, mSHP1P2 and uSHP1P2 could be predictor marker of survival, the stratification according to baseline level of circulating DNA level, mSHP1P2 and uSHP1P2 were done. The patient who had shorter OS than median OS of this study was defined as unfavorable prognosis while the patient who had longer OS was defined as favorable prognosis. The AUC-ROC was used to find out the best cutoff level to detect unfavorable prognosis group from the rest. The cut-off level of 4.5 ng ml^{-} circulating DNA level had 73% sensitivity and 71% specificity. The cut-off level of 0.7 ng ml^{-1} for mSHP1P2 had 68% sensitivity and 70% specificity. The cut-off level of 4.0 ng ml^{-} uSHP1P2 had 76% sensitivity and 60% specificity (Figure 5.5). The patients were then reclassified according to biomarker level. If the baseline value of each parameter was higher than cutoff level, the patient was classified as unfavorable prognosis. The remaining was classified as favorable prognosis.

Table 5.3 Circulating DNA level and uSHP1P2 according to each clinicopathological parameter of advanced NSCLC

Parameter	No.	Median circulating DNA levels (ng ml^{-1})	Median uSHP1P2 (ng ml^{-1})
Age			
< 60 yr	**30**	**3.4662 (0.82-39.64)**	**3.1427 (0.73-19.36)**
≥ 60 yr	**28**	**5.0434 (1.48-49.88)**	**3.9985 (1.30-24.87)**
Sex			
Male	**39**	**5.1529 (0.82-39.64)**	**3.7387 (0.73-24.87)**
Female	**19**	**3.7587 (1.33-49.88)**	**3.3333 (1.22-23.37)**
Smoking status			
Smoking	**33**	**5.2459 (1.65-25.58)**	**3.7387 (0.83-24.87)**
Non-smoking	**25**	**3.7587 (0.82-49.88)**	**3.3333 (0.73-23.37)**
ECOG status			
ECOG = 0	**6**	**4.5869 (2.16-25.58)**	**3.4590 (1.61-24.87)**
ECOG = 1	**47**	**4.2802 (0.82-49.88)**	**3.5203 (0.73-23.37)**
ECOG = 2	**5**	**5.7275 (1.48-39.64)**	**3.7387 (1.32-11.29)**
Chemotherapy			
Paclitaxel/carboplatin	**32**	**5.1994 (0.82-49.88)**	**4.0097 (0.73-24.87)**
Gemcitabine/carboplatin	**21**	**3.4838 (1.33-21.54)**	**3.1704 (0.83-19.06)**
Carboplatin	**5**	**5.7275 (1.48-21.28)**	**3.7387 (1.30-8.98)**
Pathological type			
Adenocarcinoma	**45**	**4.2793 (0.82-49.88)**	**3.4169 (0.73-24.87)**
Squamous cell CA	**6**	**4.1682 (1.65-15.66)**	**3.5586 (0.83-8.98)**
Large cell CA	**2**	**6.7067 (4.47-8.94)**	**4.9872 (3.82-6.14)**
NSCLC NOS	**5**	**4.9340 (3.01-5.71)**	**3.8489 (2.23-4.60)**
Total regimen Rx			
< 2 regimen	**37**	**5.1529 (0.82-49.88)**	**3.8265 (0.73-24.87)**
≥ 2 regimen	**21**	**4.0210 (1.33-10.40)**	**3.3917 (1.22-9.54)**

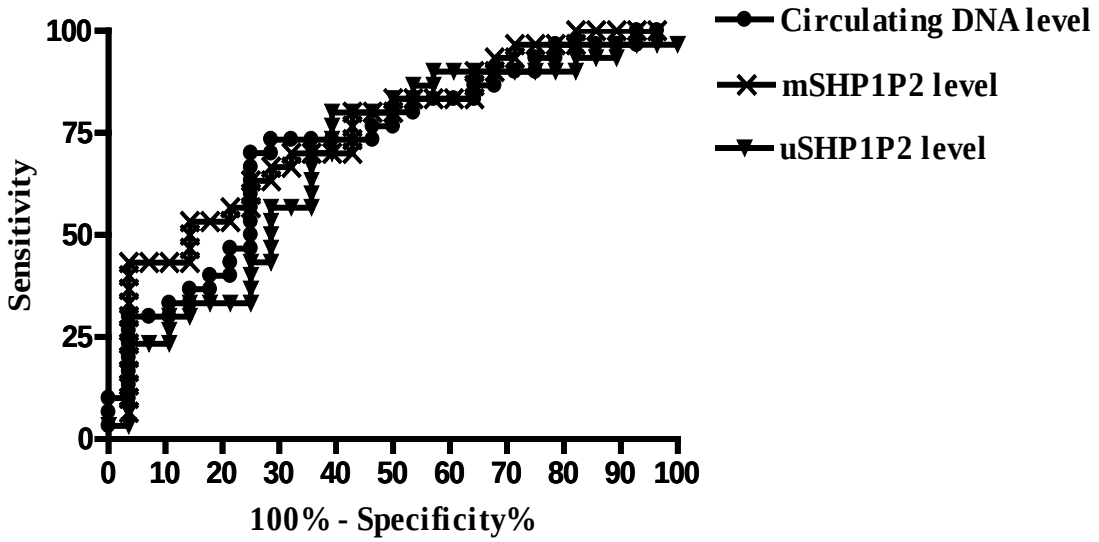


Figure 5.5 The area under the receiver operative characteristic curve by pretreatment level to detect unfavorable prognosis from favorable prognosis NSCLC

5.2.3. CIRCULATING DNA LEVEL, UNMETHYLATED SHP-1 PROMOTER 2 AND ROLE OF PREDICTOR OF SURVIVAL

The Kaplan-Meier estimates of survival with Cox proportional-hazards comparison based on baseline cut-off level of those parameters showed statistical significance to predict survival risk stratification ($p < 0.001$, 0.002 and 0.01 for circulating DNA, uSHP1P2 and mSHP1P2 respectively). The circulating DNA and mSHP1P2 also showed statistical significant to predict progression of disease ($p = 0.005$, and 0.008) but not for uSHP1P2 ($p = 0.07$). Figure 5.6 represented the OS and PFS curve of advanced NSCLC according to prognosis strategy using circulating DNA level and uSHP1P2. The OS and PFS curve according to mSHP1P2 was presented in chapter IV. Twenty-eight (48%) of patients had circulating DNA level $\geq$ 4.5 ng ml^{-1}. Median PFS of this group was 2.6 months (95% confidence interval 2.3-2.8), and median OS 4.7 months (95% confidence interval 1.5-8.0), shorter than patient who had circulating DNA level less than 4.5 ng ml^{-1}. In the favorable prognosis group median PFS was 5.6 months (95% confidence interval 4.6-6.5, $p = 0.005$) while median OS was more than 15 months ($p < 0.001$).

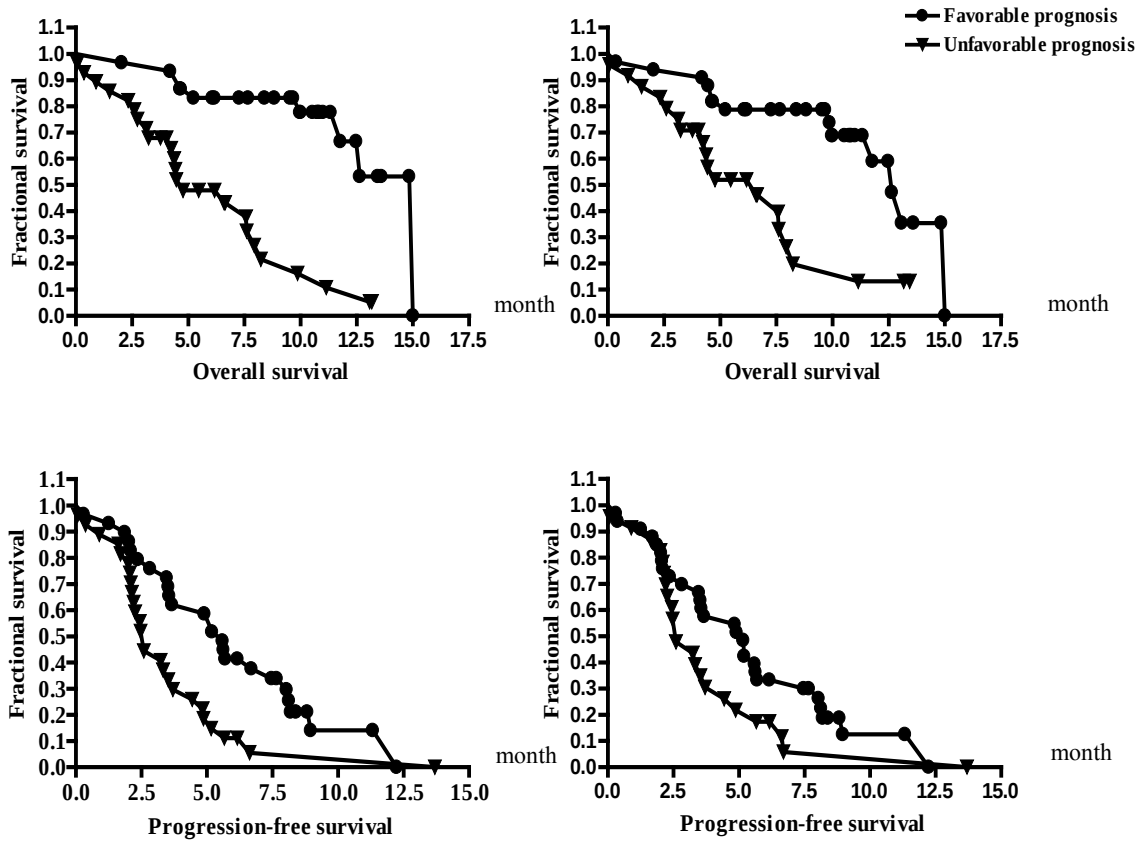


Figure 5.6 Overall survivals (above) and progression-free survival (below) in advanced NSCLC according to prognosis strategy by circulating DNA level (left panel) and plasma uSHP1P2 level (right panel)

In the aspect of unfavorable prognosis strategy according to plasma uSHP1P2 level $\geq$ 4.0_ng ml^{-1}, median OS was 6.6 months (95% confidence interval 2.6-10.5) shorter than patient who had level of plasma uSHP1P2 less than 4.0 ng ml^{-1}. Median OS of this group was 12.6 months (95% confidence interval 11.0-14.1, p = 0.002). There was the trend to shortening PFS in unfavorable prognosis group than favorable prognosis group. Median PFS was 2.6 months (95% confidence interval 1.4-3.8) in the former group and 5.1 months in the latter group, (95% confidence interval 3.4-6.8, p = 0.07) (Figure 5.6).

5.2.4. UNIVARIATE AND MULTIVARIATE ANALYSIS OF PREDICTORS OF SURVIVAL

The data from afore-mentioned showed the potential biomarker of circulating DNA level, mSHP1P2 and uSHP1P2 level. To explore whether which biomarker is

the strongest prognosis value, univariate and multivariate analysis according to Cox proportional-hazards regression analysis was done. The author also included various clinicopathological parameters that might influence the outcome such as sex, age, smoking status, Eastern Cooperative Oncology Group (ECOG) performance status and total regimen of systemic treatment. The analysis was done according to overall survival and progression-free survival. Circulating DNA level was the only parameter that had statistical significant in multivariate analysis ($p < 0.001$ for OS and 0.001 for PFS). The hazard ratio of death equal to 4.6 (95% confidence interval 1.8-11.6) if circulating DNA was more than 4.5 ng ml^{-1} (Table 5.4). The impact of circulating DNA level as predictive biomarker was influenced by the tumor-derived and non tumor-derived nucleic acid.

5.2.5. THE CORRELATION OF FOLLOW-UP CIRCULATING DNA LEVEL AND THE RESPONSE OF TREATMENT

The author explored whether early monitoring of circulating DNA level after first cycle of chemotherapy treatment was a valuable parameter to assess response of treatment. Routine clinical practice, the clinician classified response of chemotherapy treatment followed by RECIST criteria (Response Evaluation Criteria In Solid Tumor) which composed of complete response, partial response, stable disease and progressive disease. Forty follow-up blood samples before second cycle of chemotherapy were amenable to analyze according to best response of treatment by provided physician. There was a trend of increasing follow-up circulating DNA level in non response group ($p = 0.02$). The comparative analysis of pretreatment and follow-up circulating DNA level by each subgroup of response revealed the statistical significant of increasing follow-up circulating DNA level in patient who had stable disease ($p = 0.008$) but not progressive disease ($p = 0.75$). The response evaluation in this study was done by using difference imaging such as chest X-ray or CT scan which might difference from patient to patient (data not shown). Nevertheless response evaluation of advanced NSCLC seemed to correlate well with time to disease progression ($p <0.001$) but not overall survival ($p = 0.2$) (Table 5.5). This might be the effect of sequential systemic treatment which is composed of chemotherapy therapy or tyrosine kinase inhibitor. Moreover individual component

neither uSHP1P2 nor mSHP1P2 had corroborated change of level related with response of treatment.

Table 5.4 Univariate and multivariate analysis using Cox's proportional hazards regression analysis of overall survival and progression-free survival

Parameter	Univariate Hazard ratio of death (95% CI)	*p-value*	Multivariate Hazard ratio of death (95% CI)	*p-value*	Univariate Hazard ratio of recurrence (95%CI)	*p-value*	Multivariate Hazard ratio of recurrence (95%CI)	*p-value*
Age < 60 vs ≥ 60 yr	**1.1 (0.5-2.3)**	**0.7**	**1.7 (0.8-3.8)**	**0.1**	**1.0 (0.6-1.8)**	**0.8**	**1.1 (0.6-2.0)**	**0.7**
Sex male vs female	**1.2 (0.5-2.7)**	**0.5**	**0.3 (0.1-1.4)**	**0.1**	**1.0 (0.5-1.9)**	**0.8**	**0.7 (0.2-1.9)**	**0.5**
Smoking status yes vs no	**1.5 (0.7-3.2)**	**0.2**	**1.4 (0.3-5.1)**	**0.6**	**1.0 (0.5-1.7)**	**0.9**	**0.8 (0.3-2.1)**	**0.7**
ECOG 0,1 vs 2	**0.7 (0.2-2.6)**	**0.7**	**0.3 (0.07-1.5)**	**0.1**	**0.7 (0.2-1.8)**	**0.5**	**0.5 (0.1-1.8)**	**0.3**
Regimen Rx < 2 vs ≥ 2	**2.5 (1.1-5.9)**	**0.02**	**3.2 (1.1-9.1)**	**0.02**	**ND**[±]		**ND**[±]	
Circulating DNA ≥ 4.5 vs <4.5 ngml^{-1}	**2.3 (1.5-3.5)**	**< 0.001***	**4.6 (1.8-11.6)**	**0.001***	**1.5 (1.1-2.0)**	**0.007***	**1.6 (0.9-3.0)**	**0.07**
uSHP1P2 ≥ 4.0 vs < 4.0 ng ml^{-1}	**1.7 (1.2-2.5)**	**0.003**	**0.6 (0.3-1.2)**	**0.2**	**1.2 (0.9-1.7)**	**0.08**	**0.9 (0.5-1.4)**	**0.6**
mSHP1P2 0.7 vs < 0.7 ng ml^{-1}	**1.6 (1.1-2.3)**	**0.013**	**0.7 (0.4-1.3)**	**0.3**	**1.4 (1.0-1.9)**	**0.01**	**1.2 (0.8-1.7)**	**0.2**

Table 5.5 Median progression-free survival (PFS) and overall survival (OS) in advanced NSCLC with available response assessment by provided physician

Response of Rx	Number	Median PFS (month)	Median OS (month)
Partial response	16	5.6 (3.8-7.4)	11.1 (9.4-12.8)
Stable disease	18	3.5 (3.1-3.9)	9.8 (6.8-12.0)
Progressive disease	6	2.0 (1.9-2.1)	4.7 (1.0-8.4)
Overall	40	4.9 (3.7-6.0)	11.1 (7.1-15.1)

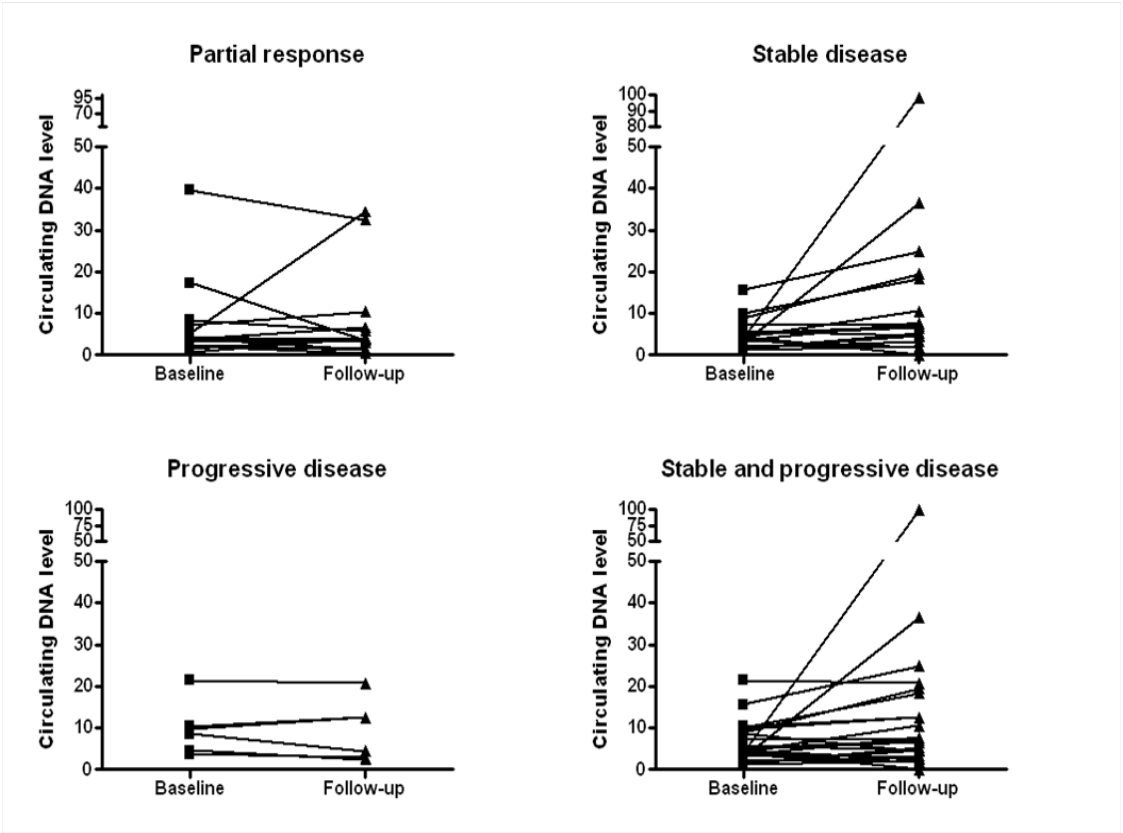


Figure 5.7 The baseline and follow-up circulating DNA level according to response of first cycle of chemotherapy treatment

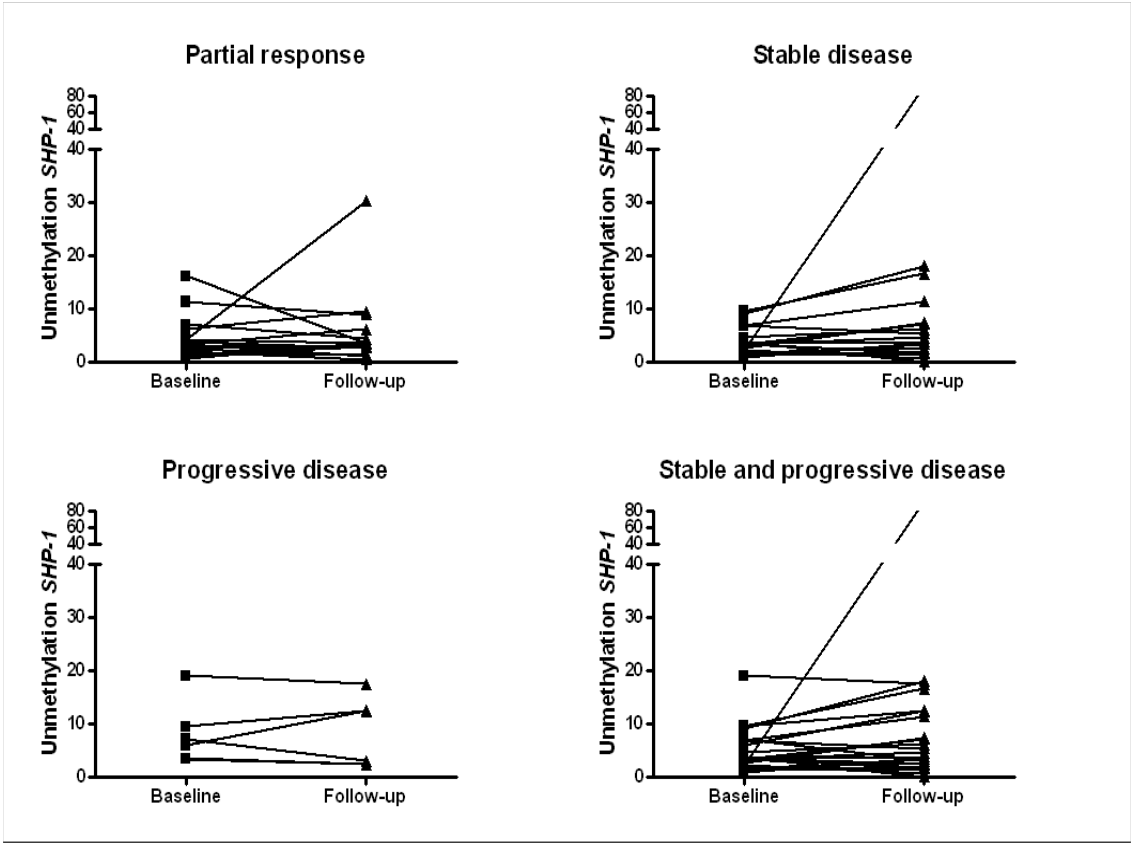


Figure 5.8 The baseline and follow-up plasma uSHP1P2 level according to response of first cycle of chemotherapy treatment

CHAPTER VI

DISCUSSION

6.1. ESTABLISH METHOD FOR SHP-1 PROMOTER 2 METHYLATION DETECTION

The first generation of methylation detection assays employed the digestion of genomic DNA with a methylation-sensitive restriction enzyme followed by either Southern blot analysis or PCR. The cleavage of DNA by restriction endonucleases HpaII is prevented by the presence of a 5-methyl group at the internal C residue of its recognition sequence CCGG. The limited availability of this method is informative restriction sites, false positivity results due to incomplete methylated sequence digestion. However contrast to previous mention; in our study the over digestion of methylation sequence limited the usefulness of this assay. This might be the result of excess HpaII restriction endonuclease in the reaction. The author used 1 unit of enzyme per 1 ng of genomic DNA, incubate for 16 hrs. One unit is defined as the amount of enzyme required to digest 1μg of λ DNA in 1 hour at 37 degree Celsius in a total reaction volume of 50 μl. The λ DNA was a duplex DNA that is isolated from the bacteriophage lambda and 48502 bps in length. No exact amount of restriction enzyme was recommended. The other possibility was potential cutting of methylated sequence. HpaII will not cut sites that have been methylated by SssI methyltransferase or HpaII Methyltransferase. However if the sites have been methylated by MspI methyltransferase, the enzyme will cut methylated sequence even though 300 times slower than unmethylated DNA and 50 times slower than partial methylation. Furthermore requirement of large amounts of high molecular weight DNA is also limited methylation assessment in fragmented plasma DNA. The author discarded this method for assessment SHP1P2 methylation.

The second generation of techniques resulted from the bisulfate treated DNA followed by PCR amplification analysis with specific primers designed to anneal with those sequences. The sequence differences resulting from various DNA methylation patterns can be revealed in difference ways such as discrimination at level of specific

primers or restriction enzyme digestion with gel electrophoresis. Even though methylation-specific PCR has previous reported as common used for methylation assessment. Subsequence techniques using specific dual hybridization probe with real-time PCR referred to the Methylight is not only specific but also sensitive and compatible with very small amounts of template DNA. The author used previous published MSP-PCR compare with our establishment methylight method. The correlation between those assays was found. However more precisely method, methylight was used to evaluate SHP1P2 methylation in this study.

The methylated-specific ARMS primers PCR method amplified non specific amplicon for SHP1P2 methylation detection. Using the concept that PCR amplification is inefficient or complete refractory if there is a mismatch between the 3` terminal nucleotide of a PCR primer and the template. These prevent the primer extension by *Taq* polymerase which lacks a 3` to 5` exonuclease activity and therefore cannot correct mismatches at the 3` terminus of the primer. The specificity or discriminating power of the 3` terminal nucleotide can be enhanced further by incorporating an additional mismatch positioned near the 3` nucleotide [81]. Comparative among nucleotide mismatch C: T mismatch which was 3` end of methylated specific primer and unmethylated sequence, was the weakest inhibitor, about 100-fold reduction if compared to a correct A, T basepair. The unexpected ease of extending the C: T mismatch was not likely to have been caused by primer-template misalignment [82]. Ayyadevara et al. also reported that the penultimate 3` nucleotide plays a minor role in mismatch discrimination [83].

6.2. EPITHELIAL SPECIFIC METHYLATION MARKER, SHP-1 PROMOTER 2, AS A PLASMA BIOMARKER

To prove the novel concept of using epithelial-specific methylation marker to detect epithelial-derived cancer, the study participants both advanced NSCLC and healthy volunteer were enrolled. Even though Yanni Y.N.et al reported the biology of circulating nucleic acid in plasma or serum of healthy individuals was predominantly hematopoietic in origin, there might be some possibility of epithelial cell-derived circulating nucleic acid. Blood circulation supply many internal epithelial cell-composed organs such as lung, liver, renal, gastrointestinal track, and genitourinary system. Sex and age match healthy volunteer was chosen owing to awareness of

gender and aging difference of methylation pattern which had been reported elsewhere [84-88]. Non-smoking status was included in the criteria of selected healthy volunteer to avoid occult medical illness related to smoking such as stroke, myocardial infarction which previous study shown abnormal elevated circulating nucleic acid [14].

The association of increased plasma DNA concentration and advanced tumor stage was reported [24]. The increased plasma DNA concentration might be the component of tumor-derived nucleic acid. In early study advanced stage disease was chosen. After explore potential role of SHP1P2 methylation in advanced NSCLC , the next step work up in early stage NSCLC should be valuable. All of advanced NSCLC participant underwent palliative chemotherapy depend on their performance status and its represent homogeneity of target population and to avoid the potential bias of clinical interpretation due to the individualized clinical management of physician.

A majority of the undetectable level of SHP1P2 methylation in healthy subjects was found (20 of 52 [38%] subjects). In contrast, in the NSCLC patients, the levels of SHP1P2 methylation were readily detectable in most of the cases (35 of 38 [92%]) with a significantly higher and broader range of methylation level. This represents amount of tumor-derived circulating nucleic acid. The result of our study is consistent with previous study which is explored tumor-specific molecular marker. From the data of advanced NSCLC only, this might not conclude the usefulness of SHP1P2 in term of diagnostic marker. However the good sensitivity and specificity of SHP1P2 testing using quantitative manner and cut-off value by area under the receiver operative curve let SHP1P2 methylation as an attractive biomarker in subsequent study to explore the potential role of diagnostic plasma biomarker. SHP1P2 methylation has potential superior to previous published circulating DNA level in term of specificity. As comparative analysis area under the receiver operative curve of SHP1P2 methylation and circulating DNA level, SHP1P2 methylation had better power of discrimination (0.93 vs 0.69). The adjusted cut-off value was incorporated to reach optimal sensitivity and specificity of measured SHP1P2 methylation.

6.3. ROLE OF SHP-1 PROMOTER 2 METHYLATION FOR PREDICTION OF SURVIVAL IN ADVANCED NON-SMALL CELL LUNG CANCER

This study is the first to demonstrate the potential role of SHP1P2, epithelial cell specific promoter methylation in plasma as a prognostic marker for advanced NSCLC. The high concentration of SHP1P2 methylation in plasma was found to be an independent prognostic marker for both progression-free survival and overall survival of advanced NSCLC patients under univariate and multivariate analyses. Instead of SHP1P2 methylation index, increasing absolute level of SHP1P2 methylation may be an indicator of tumor burden in peripheral circulation, which may represent whole-body tumor load. The higher baseline tumor volume may confer more resistance and poorer treatment outcome. However, this study could not demonstrate a good correlation of the level of SHP1P2 methylation with clinical radiographic response to treatment with chemotherapy. One possible explanation is that chemotherapy treatment may lead to the release of tumor DNA into the circulation, which may interfere with the equilibrium of circulating tumor DNA. On the other hand, our study population may not be large enough to allow the afore-mentioned analysis. Future studies should further address these issues.

6.4. THE BIOLOGY OF CIRCULATING DNA IN ADVANCED NON-SMALL CELL LUNG CANCER

Plasma SHP-1 promoter 2 methylation is a valuable molecular biomarker not only as a potential diagnostic or prognostic marker, but also usefulness in study of biology of circulating nucleic acid. The predominately circulating nucleic acid fraction derived from non-epithelial portion in healthy individual support previous evidence that it’s predominately hematopoietic in origin. However the author didn’t find the correlation between amounts of hematopoietic cell-derived nucleic acid with peripheral hematopoietic cell component. DNA degradation by plasma nucleases and related mechanism might be take part in this. Rapid clearance of plasma DNA caused the shorter life span of circulating nucleic acid than each blood component such as neutrophil life span is 24 hours where as eosinophil life span is 8-12 days. Lo YM et

al was reported the rapid clearance of circulating fetal DNA in maternal plasma by 2 hour postpartum. The mean half-life for circulating fetal DNA was 16.3 minutes (range 4-30 min) [89]. Moreover no correlation of circulating DNA level according to age or sex in healthy individual.

The significant elevated amount of circulating nucleic acid in advanced NSCLC attributed majority of tumor-derived nucleic acid and a trend of increased non tumor-derived component. Boarder range of amount of tumor-derived and non tumor-derived nucleic acid can conclude that cancer usually lead to an increase in plasma DNA but the composition varies among patient. No correlation between circulating nucleic acid and feature of primary tumor pathology such as area of necrosis, lymphoid infiltration or growth patterns [4] [37]. However the correlation between increased plasma DNA and elevated LDH level was reported [24]

In detail of each component, the author postulated that SHP1P2 methylation might correlation with tumor burden however it was difficult to assess the tumor volume of each patient owing to combine measurable and non-measurable pathology of disease. No correlation between site numbers of distant metastasis and SHP1P2 methylation level was found. The relative increased non tumor-derived nucleic acid was also not correlated with amount of peripheral hematopoietic cell. Surprisingly the positive correlation between SHP1P2 methylation (mSHP1P2) and SHP1P2 unmethylation (uSHP1P2) was confirmed from both baseline pretreatment and 3 week follow-up after first cycle of chemotherapy. The result of this experiment consisted with prior several evidences declared the interactions between host inflammatory cell and cancer.

Interaction between host immune system and tumor has been explored in various studies. Tumor microenvironment is an antitumor immune activity which is primarily mediated by the innate and adaptive immune systems through the circulating effector cells, particularly natural killer cells, neutrophils, macrophages and antigen-specific B/T lymphocytes.[90] It has been revealed as the crucial role of the host's immune system in interrupting the neoplastic phenomenon. Presence of tumor-infiltrating lymphocytes in primary tumor correlated with better clinical outcome in several types of cancer such as ovarian cancer [91], endometrial

carcinoma [92], melanoma [93], esophageal cancer [94] and colorectal cancer [95]. Nevertheless tumor escape phenomenon from the immune system had been reported either through immunosuppressive cytokines (TGF-β and IL-10) or regulatory T cells.[96, 97] Paradoxical correlation must take into account where immunity was modified by the tumor and tumor in turn was modified by the immune system led to the immunoediting concept. [98, 99] Therefore the exact origin of non tumor-derived nucleic acid is doubtful. However Jahr et al found that tumor-infiltrating T lymphocyte was not the source of circulating non tumor-derived nucleic acid. [4] Though previous reported correlation of presenting tumor-infiltrating T lymphocyte and good prognosis outcome. Then inverse correlation of prognosis and level of circulating non-tumor derived nucleic acid could be translated and supported the Jahr et al study. Several studies reported role of inflammatory cell contributed tumor promotion and progression. Tumor-associated macrophages (TAMs), the major component of infiltrate leukocyte and T regulatory cells (Tregs) which take part of tumor proliferation [100-102] might be the source of non tumor-derived nucleic acid. Furthermore bone-marrow-derived myeloid progenitors which also promote metastatic growth via inflammatory cytokine through macrophage-activating factors from metastatic carcinoma [101] are alternative hypothesis which might need further warrant.

6.5. CIRCULATING NUCLEIC ACID AND PROGNOSTIC OF ADVANCED NON-SMALL CELL LUNG CANCER, IMPLICATION FROM BOTH TUMOR AND NON-TUMOR DERIVED PROPORTION

Comparative all biomarkers state here, even though each mSHP1P2 or uSHP1P2 could be a risk adaptive molecular marker for survival and/or progression of disease. However total circulating nucleic acid, combine tumor-derived and non-tumor derived nucleic acid was the most valid molecular marker. Circulating nucleic acid was the only independent factor by Cox's proportional hazards regression analysis based upon demographic factor and all molecular markers. The author can conclude that the strongest predictive power of circulating nucleic acid was influenced from the impact of both tumor-derived and non-tumor derived nucleic acid, the tumor burden and interaction of inflammatory host cell. Prior studies of plasma DNA level in various type of malignancy such as colorectal cancer, breast

cancer, prostate cancer or non-small cell lung cancer were consisted with our reported which likely to be the same biology.

6.6. THE CORRELATION OF TREATMENT RESPONSE AND ALTERATION OF PLASMA DNA LEVEL

The major contribution of non tumor derived-nucleic acid in plasma of tumor-bearing immunocompotent mice model was explored by Garcia-Olimo D. et al. study. Significant higher level of non-tumor DNA in plasma of tumor-bearing animals was significantly was found than those in healthy animals measured by real-time PCR. However this finding was not consistent. Furthermore the large amount of non-tumor DNA level was significantly related with tumor progression [103].Gautschi O et al. study also found the correlation between elevated plasma DNA level and tumor progression after chemotherapy in advanced NSCLC. The author found the trend of increasing follow-up circulating DNA level and non-response group after treatment by provided physician. Tissue necrosis from both tumor and inflammatory cell components might explain this phenomenon. The circulating nucleic acid is the unique biomarker to represent progression of disease. The important of non tumor-derived circulating nucleic acid was still overlooked. The discovery of this information might lead to novel management.

CHAPTER VII

CONCLUSION

SUMMARY THE WHOLE THESIS

Our study prove the concept of using epithelial specific methylation, SHP1 promoter 2 methylation, as a plasma biomarker to represent nucleic acid component form epithelial-derived cancer cell. In here the author used non-small cell lung cancer, the most lethal disease as a model. SHP1 promoter 2 methylation level was detected higher and boarder range in plasma of advanced non-small cell lung cancer but not in sex and age match healthy volunteer. Using cut-off value had better sensitivity and specificity of molecular assay to discriminate both condition. This was represented usefulness in diagnosis potential. The level of SHP1 promoter 2 methylation was also represented risk stratification according to progression of disease and survival outcome.

SHP-1 promoter 2 methylation and unmethylation portion could represent tumor biology of circulating nucleic acid in both healthy volunteer and advanced stage non-small cell lung cancer. Predominate unmethylation fraction in plasma of healthy volunteer represented the hematopoietic origin of circulating nucleic acid. Total amount of circulating nucleic acid as the combination of tumor-derived and inflammatory cell-derived was increased in plasma of advanced non-small cell lung cancer and it was also the valuable predictive biomarker of survival and response of treatment. Increased tumor burdens composed of both cancer and infiltrative inflammatory cell or the interaction between those two components might have role.

LIMITATION OF THE RESEARCH

Even though this thesis explored the role of SHP-1 promoter 2 methylation as a valuable molecular marker, further extended study participants in early stage disease might confirmed the diagnostic role. Moreover the comparative data between same disease stage with or without treatment might let us know more information of clinical interpretation of this molecular marker.

PROVIDING SUGGESTING FOR FURTHER RESEARCH EFFORT

SHP-1 promoter 2 methylation was first proved as useful molecular marker in advanced non-small cell lung cancer. However the same application of this molecular marker in various type of epithelial cancer was the challenging issue. SHP-1 promoter 2 might be a universal molecular marker for detection various epithelial cancer cell derived nucleic acid such as breast cancer, colon cancer or colon cancer.

RESEARCH BENEFITS AND APPLICATIONS

The biomarker risk stratification of EBV DNA detection level has been first incorporated in UICC clinical staging of nasopharyngeal cancer, adjusted clinical management study according to biomarker risk stratification is going on. The further validate clinical usefulness of SHP-1 promoter 2 methylation in various stages of non-small cell lung cancer might lead to the same implication. Moreover potential role of SHP-1 promoter 2 methylation as a diagnostic marker has open-up challenging issue in cancer molecular marker study.

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APPENDICES

APPENDIX A

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	**เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย** (Information sheet for research participant)

ชื่อโครงการวิจัย การตรวจหาสภาวะเหนือพันธุกรรมของยีน *SHP-1* promoter 2 โดยการเติมหมู่เมททิลในพลาสม่าของผู้ป่วยมะเร็งปอดและบทบาททางคลีนิค

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เรียน ผู้เข้าร่วมโครงการวิจัยทุกท่าน

ท่านได้รับเชิญให้เข้าร่วมในโครงการวิจัยนี้เนื่องจากท่านเป็นผู้ป่วยโรคมะเร็งปอดชนิดเซลไม่เล็กระยะลุกลามที่ได้รับการรักษาด้วยยาเคมีบำบัด ก่อนที่ท่านจะตัดสินใจเข้าร่วมในการศึกษาวิจัยดังกล่าว ขอให้ท่านอ่านเอกสารฉบับนี้อย่างถี่ถ้วน เพื่อให้ท่านได้ทราบถึงเหตุผลและรายละเอียดของการศึกษาวิจัยในครั้งนี้ หากท่านมีข้อสงสัยใดๆ เพิ่มเติม กรุณาซักถามจากทีมงานของแพทย์ผู้ทำวิจัย หรือแพทย์ผู้ร่วมทำวิจัยซึ่งจะเป็นผู้สามารถตอบคำถามและให้ความกระจ่างแก่ท่านได้

ท่านสามารถขอคำแนะนำในการเข้าร่วมโครงการวิจัยนี้จากครอบครัว เพื่อน หรือแพทย์ประจำตัวของท่านได้ ท่านมีเวลาอย่างเพียงพอในการตัดสินใจโดยอิสระ ถ้าท่านตัดสินใจแล้วว่าจะเข้าร่วมในโครงการวิจัยนี้ ขอให้ท่านลงนามในเอกสารแสดงความยินยอมของโครงการวิจัยนี้

<u>เหตุผลความเป็นมา</u>

การตรวจหาสารพันธุกรรมเซลมะเร็งในสารประกอบของเลือดในผู้ป่วยโรคมะเร็งได้มีการศึกษาวิจัย ว่าสามารถช่วยพยากรณ์โรค รวมถึงประโยชน์ต่อการพิจารณาการรักษาในผู้ป่วยโรคมะเร็งปอดชนิดเซลไม่เล็กระยะลุกลาม หากแต่ยังไม่สามารถนำมาพัฒนาเพื่อใช้ได้จริงทางคลินิก ข้อจำกัดที่สำคัญสืบเนื่องจากความไวและความจำเพาะของการตรวจนั้นๆจึงเป็นที่มาของการศึกษาเพื่อทำการตรวจหาสภาวะเหนือพันธุกรรมของยีน *SHP-1* promoter 2 ซึ่งเป็นสภาวะที่ความจำเพาะกับเซลมะเร็งชนิดเยื่อบุ ว่าจะมีความไว/ความจำเพาะกับผู้ป่วยโรคมะเร็งปอดชนิดเซลไม่เล็ก และจะสามารถใช้ในการพยากรณ์โรคได้หรือไม่ โดยทำการศึกษาร่วมกับข้อมูลทางคลีนิก

<u>วัตถุประสงค์ของการศึกษา</u>

วัตถุประสงค์หลักจากการศึกษาในครั้งนี้คือ ศึกษาว่าการตรวจหาสภาวะเหนือพันธุกรรมของยีน *SHP-1* promoter 2 ในสารประกอบเลือดของผู้ป่วยมะเร็งปอดชนิดเซลไม่เล็กระยะลุกลามจะสามารถใช้พยากรณ์โรคในผู้ป่วยที่ได้รับการรักษาด้วยยาเคมีบำบัด ร่วมกับข้อมูลทางคลินิก จำนวนผู้เข้าร่วมในโครงการวิจัย คือ 50 คน

 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	**เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย** (Information sheet for research participant)

<u>วิธีการที่เกี่ยวข้องกับการวิจัย</u>

หากท่านมีคุณสมบัติตามเกณฑ์คัดเข้า ท่านจะได้รับเชิญให้มาพบแพทย์ตามวันเวลาที่ผู้ทำวิจัยนัดหมาย คือ ตลีนิกเคมีบำบัด ตึกว่องวานิช ชั้น 4 เวลา 9.00-12.00 เพื่อ สอบถามประวัติที่เกี่ยวข้อง ตรวจสุขภาพร่างกายอย่างละเอียด ประเมินผลทางห้องปฏิบัติการต่าง ๆ โดยตลอดระยะเวลาที่ท่านอยู่ในโครงการวิจัย คือ 2 เดือน และมาพบแผนวิจัยหรือแพทย์ผู้รักษาร่วมในโครงการวิจัยทั้งสิ้น 3 ครั้ง

การวิจัยนี้เป็นการติดตามผู้ป่วยมะเร็งปอดชนิดเซลไม่เล็กระยะลุกลามที่ได้รับการรักษาด้วยยาเคมีบำบัดสูตรมาตรฐาน ซึ่งผู้วิจัยจะทำการเก็บตัวอย่างเลือด 10 มิลลิลิตร 3 ครั้งห่างกันทุก 3 สัปดาห์ ก่อนการให้ยาเคมีบำบัดและพร้อมเริ่มการรักษาด้วยยาเคมีบำบัดเพื่อทำการตรวจหาสารพันธุกรรมเซลมะเร็งปอดชนิดเซลไม่เล็กกล่าวคือสภาวะเหนือพันธุกรรมซึ่งเติมหมู่เมททิลของยีน *SHP-1* promoter 2 และ/หรือการตรวจสารพันธุกรรมอื่นๆที่เกี่ยวข้องในการศึกษาต่อๆไป นอกจากนี้ผู้วิจัยจะทำการเก็บข้อมูลทางคลีนิกของผู้ป่วยที่เกี่ยวข้องร่วมด้วย

<u>ความรับผิดชอบของอาสาสมัครผู้เข้าร่วมในโครงการวิจัย</u>

เพื่อให้งานวิจัยนี้ประสบความสำเร็จ ผู้ทำวิจัยใคร่ขอความความร่วมมือจากท่าน โดยจะขอให้ท่านปฏิบัติตามคำแนะนำของผู้ทำวิจัยอย่างเคร่งครัด รวมทั้งแจ้งอาการผิดปกติต่าง ๆ ที่เกิดขึ้นกับท่านระหว่างที่ท่านเข้าร่วมในโครงการวิจัยให้ผู้ทำวิจัยได้รับทราบ

เพื่อความปลอดภัย ท่านไม่ควรใช้วัคซีน หรือรับประทานยาอื่น จากการจ่ายยาโดยแพทย์อื่นหรือซื้อยาจากร้านขายยา ดังนั้นขอให้ท่านแจ้งผู้ทำวิจัยเกี่ยวกับยาที่ท่านได้รับในระหว่างที่ท่านอยู่ในโครงการวิจัย

<u>ความเสี่ยงที่ได้รับจากการเจาะเลือด</u>

ท่านมีโอกาสที่จะเกิดอาการเจ็บ เลือดออก ช้ำจากการเจาะเลือด อาการบวมบริเวณที่เจาะเลือดหรือหน้ามืด และโอกาสที่จะเกิดการติดเชื้อบริเวณที่เจาะเลือดพบได้น้อยมาก

<u>ความเสี่ยงที่ไม่ทราบแน่นอน</u>

ท่านอาจเกิดอาการข้างเคียง หรือความไม่สบาย นอกเหนือจากที่ได้แสดงในเอกสารฉบับนี้ ซึ่งอาการข้างเคียงเหล่านี้เป็นอาการที่ไม่เคยพบมาก่อน เพื่อความปลอดภัยของท่าน ควรแจ้งผู้ทำวิจัยให้ทราบทันทีเมื่อเกิดความผิดปกติใดๆ เกิดขึ้น

หากท่านมีข้อสงสัยใดๆ เกี่ยวกับความเสี่ยงที่อาจได้รับจากการเข้าร่วมในโครงการวิจัย ท่านสามารถสอบถามจากผู้ทำวิจัยได้ตลอดเวลา

หากมีการค้นพบข้อมูลใหม่ ๆ ที่อาจมีผลต่อความปลอดภัยของท่านในระหว่างที่ท่านเข้าร่วมในโครงการวิจัย ผู้ทำวิจัยจะแจ้งให้ท่านทราบทันที เพื่อให้ท่านตัดสินใจว่าจะอยู่ในโครงการวิจัยต่อไปหรือจะขอถอนตัวออกจากการวิจัย

 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย (Information sheet for research participant)

<u>การพบแพทย์นอกตารางนัดหมายในกรณีที่เกิดอาการข้างเคียง</u>

หากมีอาการข้างเคียงใด ๆ เกิดขึ้นกับท่าน ขอให้ท่านรีบมาพบแพทย์ที่สถานพยาบาลทันที ถึงแม้ว่าจะอยู่นอกตารางการนัดหมาย เพื่อแพทย์จะได้ประเมินอาการข้างเคียงของท่าน และให้การรักษาที่เหมาะสมทันที หากอาการดังกล่าวเป็นผลจากการเข้าร่วมในโครงการวิจัย ท่านจะไม่เสียค่าใช้จ่าย

<u>ประโยชน์ที่อาจได้รับ</u>

การเข้าร่วมในโครงการวิจัยนี้ไม่ได้มีผลต่อการรักษาของผู้ป่วยซึ่งได้รับการรักษาด้วยยาเคมีบำบัดสูตรมาตรฐาน ท่านจะไม่ได้รับประโยชน์ใดๆจากการเข้าร่วมในการวิจัยครั้งนี้ แต่ผลการศึกษาที่ได้จะสามารถพัฒนาการดูแลผู้ป่วยโรคมะเร็งปอดชนิดเซลไม่เล็กระยะลุกลามต่อไป

<u>วิธีการและรูปแบบการรักษาอื่น ๆ ซึ่งมีอยู่สำหรับอาสาสมัคร</u>

ท่านไม่จำเป็นต้องเข้าร่วมโครงการวิจัยนี้เพื่อประโยชน์ในการรักษาโรคที่ท่านเป็นอยู่ เนื่องจากการรักษาที่ท่านได้รับเป็นการรักษาตามมาตรฐาน โครงการวิจัยนี้เป็นเพียงการศึกษาติดตามผลข้างเคียงที่อาจจะเกิดขึ้นได้จากการรักษา

<u>ข้อปฏิบัติของท่านขณะที่ร่วมในโครงการวิจัย</u>

ขอให้ท่านปฏิบัติดังนี้

- ขอให้ท่านให้ข้อมูลทางการแพทย์ของท่านทั้งในอดีต และปัจจุบัน แก่ผู้ทำวิจัยด้วยความสัตย์จริง
- ขอให้ท่านแจ้งให้ผู้ทำวิจัยทราบความผิดปกติที่เกิดขึ้นระหว่างที่ท่านร่วมในโครงการวิจัย
- ขอให้ท่านงดการใช้ยาอื่นนอกเหนือจากที่ผู้ทำวิจัยได้จัดให้ รวมถึงการรักษาอื่น ๆ เช่น การรักษาด้วยสมุนไพร การซื้อยาจากร้านขายยา
- ขอให้ท่านแจ้งให้ผู้ทำวิจัยทราบทันที หากท่านได้รับยาอื่นนอกเหนือจากยาที่ใช้ในการศึกษาตลอดระยะเวลาที่ท่านอยู่ในโครงการวิจัย
- ขอให้ท่านนำยาที่ใช้ในการศึกษาของท่านทั้งหมดที่เหลือจากการรับประทานมาให้ผู้ทำวิจัยทุกครั้งที่นัดหมายให้มาพบ

<u>อันตรายที่อาจเกิดขึ้นจากการเข้าร่วมในโครงการวิจัยและความรับผิดชอบของผู้ทำวิจัย/ผู้สนับสนุนการวิจัย</u>

หากพบอันตรายที่เกิดขึ้นจากการวิจัย ท่านจะได้รับการรักษาอย่างเหมาะสมทันที หากพิสูจน์ได้ว่าท่านปฏิบัติตามคำแนะนำของทีมผู้ทำวิจัยแล้ว ผู้ทำวิจัย /ผู้สนับสนุนการวิจัยยินดีจะรับผิดชอบค่าใช้จ่ายในการรักษาพยาบาลของท่าน และการลงนามในเอกสารให้ความยินยอม ไม่ได้หมายความว่าท่านได้สละสิทธิ์ทางกฎหมายตามปกติที่ท่านพึงมี

ในกรณีที่ท่านได้รับอันตรายใด ๆ หรือต้องการข้อมูลเพิ่มเติมที่เกี่ยวข้องกับโครงการวิจัย ท่านสามารถ

 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย (Information sheet for research participant)

ติดต่อกับผู้ทำวิจัยคือ พญ.ชนิดา วินะยานุวัติคุณได้ตลอด 24 ชั่วโมง

<u>การเข้าร่วมและการสิ้นสุดการเข้าร่วมโครงการวิจัย</u>

การเข้าร่วมในโครงการวิจัยครั้งนี้เป็นไปโดยความสมัครใจ หากท่านไม่สมัครใจจะเข้าร่วมการศึกษาแล้ว ท่านสามารถถอนตัวได้ตลอดเวลา การขอถอนตัวออกจากโครงการวิจัยจะไม่มีผลต่อการดูแลรักษาโรคของท่านแต่อย่างใด

ผู้ทำวิจัยอาจถอนท่านออกจากการเข้าร่วมการวิจัย เพื่อเหตุผลด้านความปลอดภัยของท่าน หรือเมื่อผู้สนับสนุนการวิจัยยุติการดำเนินงานวิจัย หรือ ในกรณีดังต่อไปนี้

- ท่านไม่สามารถปฏิบัติตามคำแนะนำของผู้ทำวิจัย
    - ท่านรับประทานยาที่ไม่อนุญาตให้ใช้ในการศึกษา
    - ท่านตั้งครรภ์ระหว่างที่เข้าร่วมโครงการวิจัย
    - ท่านเกิดอาการข้างเคียง หรือความผิดปกติของผลทางห้องปฏิบัติการจากการได้รับยาที่ใช้ในการศึกษา
    - ท่านแพ้ยาที่ใช้ในการศึกษา
    - ท่านต้องการปรับเปลี่ยนการรักษาด้วยยาตัวที่ไม่ได้รับอนุญาตจากการวิจัยครั้งนี้

<u>การปกป้องรักษาข้อมูลความลับของอาสาสมัคร</u>

ข้อมูลที่อาจนำไปสู่การเปิดเผยตัวท่าน จะได้รับการปกปิดและจะไม่เปิดเผยแก่สาธารณชน ในกรณีที่ผลการวิจัยได้รับการตีพิมพ์ ชื่อและที่อยู่ของท่านจะต้องได้รับการปกปิดอยู่เสมอ โดยจะใช้เฉพาะรหัสประจำโครงการวิจัยของท่าน

จากการลงนามยินยอมของท่านผู้ทำวิจัย และผู้สนับสนุนการวิจัยสามารถเข้าไปตรวจสอบบันทึกข้อมูลทางการแพทย์ของท่านได้แม้จะสิ้นสุดโครงการวิจัยแล้วก็ตาม หากท่านต้องการยกเลิกการให้สิทธิ์ดังกล่าว ท่านสามารถแจ้ง หรือเขียนบันทึกขอยกเลิกการให้คำยินยอม โดยส่งไปที่ 1873 หน่วยมะเร็งวิทยา ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์ มหาวิทยาลัย ถ. อังรีดูนังต์ แขวงพระนคร เขตปทุมวัน กทม. 10330

หากท่านขอยกเลิกการให้คำยินยอมหลังจากที่ท่านได้เข้าร่วมโครงการวิจัยแล้ว ข้อมูลส่วนตัวของท่านจะไม่ถูกบันทึกเพิ่มเติม อย่างไรก็ตามข้อมูลอื่น ๆ ของท่านอาจถูกนำมาใช้เพื่อประเมินผลการวิจัย และท่านจะไม่สามารถกลับมาเข้าร่วมในโครงการนี้ได้อีก ทั้งนี้เนื่องจากข้อมูลของท่านที่จำเป็นสำหรับใช้เพื่อการวิจัยไม่ได้ถูกบันทึก

จากการลงนามยินยอมของท่านแพทย์ผู้ทำวิจัยสามารถบอกรายละเอียดของท่านที่เกี่ยวกับการเข้าร่วมโครงการวิจัยนี้ให้แก่แพทย์ผู้รักษาท่านได้

<u>สิทธิ์ของผู้เข้าร่วมในโครงการวิจัย</u>

 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	**เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย** (Information sheet for research participant)

ในฐานะที่ท่านเป็นผู้เข้าร่วมในโครงการวิจัย ท่านจะมีสิทธิ์ดังต่อไปนี้

1. ท่านจะได้รับทราบถึงลักษณะและวัตถุประสงค์ของการวิจัยในครั้งนี้
2. ท่านจะได้รับการอธิบายเกี่ยวกับระเบียบวิธีการของการวิจัยทางการแพทย์ รวมทั้งยาและอุปกรณ์ที่ใช้ในการวิจัยครั้งนี้
3. ท่านจะได้รับการอธิบายถึงความเสี่ยงและความไม่สบายที่จะได้รับจากการวิจัย
4. ท่านจะได้รับการอธิบายถึงประโยชน์ที่ท่านอาจจะได้รับจากการวิจัย
5. ท่านจะได้รับการเปิดเผยถึงทางเลือกในการรักษาด้วยวิธีอื่น ยา หรืออุปกรณ์ซึ่งมีผลดีต่อท่านรวมทั้งประโยชน์และความเสี่ยงที่ท่านอาจได้รับ
6. ท่านจะได้รับทราบแนวทางในการรักษา ในกรณีที่พบโรคแทรกซ้อนภายหลังการเข้าร่วมในโครงการวิจัย
7. ท่านจะมีโอกาสได้ซักถามเกี่ยวกับงานวิจัยหรือขั้นตอนที่เกี่ยวข้องกับงานวิจัย
8. ท่านจะได้รับทราบว่าการยินยอมเข้าร่วมในโครงการวิจัยนี้ ท่านสามารถขอถอนตัวจากโครงการเมื่อไรก็ได้ โดยผู้เข้าร่วมในโครงการวิจัยสามารถขอถอนตัวจากโครงการโดยไม่ได้รับผลกระทบใด ๆ ทั้งสิ้น
9. ท่านจะได้รับสำเนาเอกสารใบยินยอมที่มีทั้งลายเซ็นและวันที่
10. ท่านจะได้โอกาสในการตัดสินใจว่าจะเข้าร่วมในโครงการวิจัยหรือไม่ก็ได้ โดยปราศจากการใช้อิทธิพลบังคับข่มขู่ หรือการหลอกลวง

หากท่านไม่ได้รับการชดเชยอันควรต่อการบาดเจ็บหรือเจ็บป่วยที่เกิดขึ้นโดยตรงจากการวิจัย หรือท่านไม่ได้รับการปฏิบัติตามที่ปรากฏในเอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในการวิจัย ท่านสามารถร้องเรียนได้ที่ คณะกรรมการจริยธรรมการวิจัย คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ตึกอานันทมหิดลชั้น 3 โรงพยาบาลจุฬาลงกรณ์ ถนนพระราม 4 ปทุมวัน กรุงเทพฯ 10330 โทร 0-2256-4455 ต่อ 14, 15 ในเวลาราชการ

ขอขอบคุณในการร่วมมือของท่านมา ณ ที่นี้

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APPENDIX B

 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	**เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย** (Information sheet for research participant)

การวิจัยเรื่อง การตรวจหาสภาวะเหนือพันธุกรรมของยีน *SHP-1* promoter 2 โดยการเติมหมู่ เมททิลใน พลาสม่าของผู้ป่วยมะเร็งปอดและบทบาททางคลีนิค

วันให้คำยินยอม วันที่.....เดือน.....พ.ศ.....

ข้าพเจ้า นาย/นาง/นางสาว..... ....ที่อยู่ .....ได้อ่านรายละเอียดจากเอกสารข้อมูลสำหรับผู้เข้าร่วมโครงการวิจัยวิจัยที่แนบมาฉบับวันที่ 10 มิถุนายน 2552 และข้าพเจ้ายินยอมเข้าร่วมโครงการวิจัยโดยสมัครใจ

ข้าพเจ้าได้รับสำเนาเอกสารแสดงความยินยอมเข้าร่วมในโครงการวิจัยที่ข้าพเจ้าได้ลงนาม และ วันที่ พร้อมด้วยเอกสารข้อมูลสำหรับผู้เข้าร่วมโครงการวิจัย ทั้งนี้ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย ระยะเวลาของการทำวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัย และแนวทางรักษาโดยวิธีอื่นอย่างละเอียด ข้าพเจ้ามีเวลาและโอกาสเพียงพอในการซักถามข้อสงสัยจนมีความเข้าใจอย่างดีแล้ว โดยผู้วิจัยได้ตอบคำถามต่าง ๆ ด้วยความเต็มใจไม่ปิดบังซ่อนเร้นจนข้าพเจ้าพอใจ

ข้าพเจ้ารับทราบจากผู้วิจัยว่าหากเกิดอันตรายใด ๆ จากการวิจัยดังกล่าว ผู้เข้าร่วมวิจัยจะได้รับการรักษาพยาบาลโดยไม่เสียค่าใช้จ่าย แต่ไม่ได้รับค่าชดเชยนอกเหนือจากค่ารักษาพยาบาล

ข้าพเจ้ามีสิทธิที่จะบอกเลิกเข้าร่วมในโครงการวิจัยเมื่อใดก็ได้ โดยไม่จำเป็นต้องแจ้งเหตุผล และการบอกเลิกการเข้าร่วมการวิจัยนี้ จะไม่มีผลต่อการรักษาโรคหรือสิทธิอื่น ๆ ที่ข้าพเจ้าจะพึงได้รับต่อไป

ผู้วิจัยรับรองว่าจะเก็บข้อมูลส่วนตัวของข้าพเจ้าเป็นความลับ และจะเปิดเผยได้เฉพาะเมื่อได้รับการยินยอมจากข้าพเจ้าเท่านั้น บุคคลอื่นในนามของบริษัทผู้สนับสนุนการวิจัย คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน สำนักงานคณะกรรมการอาหารและยาอาจได้รับอนุญาตให้เข้ามาตรวจและประมวลข้อมูลของผู้เข้าร่วมวิจัย ทั้งนี้จะต้องกระทำไปเพื่อวัตถุประสงค์เพื่อตรวจสอบความถูกต้องของข้อมูลเท่านั้น โดยการตกลงที่จะเข้าร่วมการศึกษานี้ข้าพเจ้าได้ให้คำยินยอมที่จะให้มีการตรวจสอบข้อมูลประวัติทางการแพทย์ของผู้เข้าร่วมวิจัยได้

ผู้วิจัยรับรองว่าจะไม่มีการเก็บข้อมูลใด ๆ ของผู้เข้าร่วมวิจัย เพิ่มเติม หลังจากที่ข้าพเจ้าขอยกเลิกการเข้าร่วมโครงการวิจัยและต้องการให้ทำลายเอกสารและ/หรือ ตัวอย่างที่ใช้ตรวจสอบทั้งหมดที่สามารถสืบค้นถึงตัวข้าพเจ้าได้

ข้าพเจ้าเข้าใจว่า ข้าพเจ้ามีสิทธิ์ที่จะตรวจสอบหรือแก้ไขข้อมูลส่วนตัวของข้าพเจ้าและสามารถยกเลิกการให้สิทธิในการใช้ข้อมูลส่วนตัวของข้าพเจ้าได้ โดยต้องแจ้งให้ผู้วิจัยรับทราบ

ข้าพเจ้าได้ตระหนักว่าข้อมูลในการวิจัยรวมถึงข้อมูลทางการแพทย์ของข้าพเจ้าที่ไม่มีการเปิดเผยชื่อ จะผ่านกระบวนการต่าง ๆ เช่น การเก็บข้อมูล การบันทึกข้อมูลในแบบบันทึกและในคอมพิวเตอร์ การตรวจสอบ

 คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย	**เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย** (Information sheet for research participant)

การวิเคราะห์ และการรายงานข้อมูลเพื่อวัตถุประสงค์ทางวิชาการ รวมทั้งการใช้ข้อมูลทางการแพทย์ในอนาคต หรือการวิจัยทางด้านเภสัชภัณฑ์ เท่านั้น

ข้าพเจ้าได้อ่านข้อความข้างต้นและมีความเข้าใจดีทุกประการแล้ว ยินดีเข้าร่วมในการวิจัยด้วยความเต็มใจ จึงได้ลงนามในเอกสารแสดงความยินยอมนี้

.....ลงนามผู้ให้ความยินยอม

(.....) ชื่อผู้ยินยอมตัวบรรจง

วันที่ .....เดือน.....พ.ศ.....

ข้าพเจ้าได้อธิบายถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตราย หรืออาการไม่พึงประสงค์หรือความเสี่ยงที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียด ให้ผู้เข้าร่วมในโครงการวิจัยตามนามข้างต้นได้ทราบและมีความเข้าใจดีแล้ว พร้อมลงนามลงในเอกสารแสดงความยินยอมด้วยความเต็มใจ

.....ลงนามผู้ทำวิจัย

(.....) ชื่อผู้ทำวิจัย ตัวบรรจง

วันที่ .....เดือน.....พ.ศ.....

.....ลงนามพยาน

(.....) ชื่อพยาน ตัวบรรจง

วันที่ .....เดือน.....พ.ศ.....

APPENDIX C

PROBE AND PRIMER SEQUENCE

GAPDH PROBE AND PRIMER SEQUNCE

LOCUS NC_000012 3880 bp DNA linear CON 03-MAR-2008

ACCESSION _000012 REGION: 6513918..6517797

VERSION NC_000012.10 GI:89161190

2881 gtatcgtgga aggactcatg gtatgagagc tggggaatgg **cccacctttc**

2941 ctggctcctc cctgccgggg **ctgcgtgcaa ccctggggtt** ggggttctg

3001 gggactggct ttcccataat ttcctttcaa ggtggggagg gaggtagagg ggtgatgtgg

3061 g**gagtacgct gcagggcctc a**ctcctttg cagaccacag tccatgccat cactgccacc

GAPDH2924f	5`-tgaggctcccacctttctcatc-3`
GAPDH3081r(ivs)	5`-tgaggccctgcagcgtactc-3`
GAPDH2969Taqman	5`-VIC-gctgcgtgcaaccctggggtt-TAMPRA-3`

SHP-1 PROMOTER 2 METHYLATION PROBE AND PRIMER SEQUNCE

162901 agatagcccc tgtttcatag ggctgtggtg agaaaccaat cagacaaggc atgtgaacgc
162961 cattatagca cagcgcccgg catccagcag gactcactcg atgacagttg tcaccgccat
163021 cattgttatt agcgtgggcc agggagggct gcgtaaaagc agctggtgga ggagggagag
163081 atgccgtggg accgtctggg ttcgcatgcg tgaagtatta tctgggcctg gagtgtgcaa
163141 ggcacacatg tgtccttact gcatgtgttg tcacatatgt gcaatgccat gctcctgagc

REAL-TIME PCR PROBE AND PRIMER

Methylation sequence

agatagTTTTt gtttTatagggTtgtggtgagaaaTT aatTagaTaaggTatgtgaaCGT
TattatagTaTagCGTTCGgTatTTagTaggaTtTaTtCGatgaTagttgtTaTCGTTat
Tattgttattag**CG**tgggTTagggagggTtgCGtaaaagTagTt **ggtggaggagggagag**
atgT .
ttaTtgTatgtgttgtTaTatatgtgTaatgTTatgTtTTtgagT

Unmethylation sequence

Tattgttattag**TG**tgggTTagggagggTtgTGtaaaagTagTt **ggtggaggagggagag**
atgT **TTtggagtgtgTaa**
ttaTtgTatgtgttgtTaTatatgtgTaatgTTatgTtTTtgagT

T = END FORWARD PRIMER
TT = END REVERSE PRIMER

SHP1TAQF 5`-ggTggAggAgggAgAgATgT-3`
SHP1TAQR 5`-AACACATATATACCTTACACACTCCAAA-3`
RMETSHP1PROBE 5`-VIC-ACgAACCCAAACgATCCCACg-TAMRA-3`
RUNMETSHP1PROBE5`FAM-CACATACAAACCCAAACAATCCCACA-TAMRA-3`

METHYLATION-SPECIFIC PCR PRIMER

Methylation sequence

agatagTTTTt gtttTatagggTtgtggtgagaaaTT aatTagaTaaggTatgtgaaCGT

TattatagTa TagCGTTCGg TatTTagTaggaTtTaTtCGatgaTagttgtTaTCGTTat

TattgttattagCGtgggTTagggagggTtgCGtaaaagTagTtggtggaggagggagag

atgT TTtggagtgtgTaa

ttaTtgTatgtgttgtTaTatatgtgTaatgTTatgTtTTtgag

Unmethylation sequence

agatagtttt tgttttatag ggttgtggtg agaaattaat tagataaggt atgtgaatgt

tattatagta tagtgtttgg tatttagtag gatttatttg atgatagttg ttattgttat

TattgttattagTGtgggTTagggagggTtgTGtaaaagTagTtggtggaggagggagag

atgT TTtggagtgtgTaa

ttaTtgTatgtgttgtTaTatatgtgTaatgTTatgTtTTtgagT

Methylation specific primer

5′-TGT-GAA-CGT-TAT-TAT-AGT-ATA-GCG-3′ and

5′-CCA-AAT-AAT-ACT-TCA-CGC-ATA-CG-3

Unmethylation specific primer

5′-GTG-AAT-GTT-ATT-ATA-GTA-TAG-TGT-TTG-G-3′ and

5′-TTC-ACA-CAT-ACA-AAC-CCA-AAC-AAT-3′.

PRIMER FOR Hpa II ENZYME RESTRICTION

gtgggaccgtctgggttcgcatgcgtgaagtattatctgggcctggagtgtgcaaggcac

acatgtgtccttactgcatgtgttgtcacatatgtgcaatgccatgctcctgagcctttg

attgcagacgtgtgggaagtgggccccgtccccacccccagtgccaccctgctctgcttc

tcttcccttgctgtgctctaaaacgagaagtacaagtgagttcccccaaggggtcggccg

cgcctcttcctgtccccgccctgccggctgccccaggccagtggagtggcagccccagaa

ctgggaccaccggggggtggtgaggcggccccggcactgggagctgcatctgaggcttag

tccctgagctctctgcctgcccagactagctgcacctcctca

SHP1-p2forward	5`- CTCTTCCTgTCCCCgCCCT -3`
SHP1-p2reverse	5`-TAAgCCTCAgATgCAgCTCCCAgT-3`

AMPLIFICATION REFRACTORY MUTATION SYSTEMS PRIMER

Methylation sequence

agatagTTTTt gtttTatagggTtgtggtgagaaaTT aatTagaTaaggTatgtgaaCGT

TattatagTa TagCGTTCGg TatTTagTaggaTtTaTtCGatgaTagttgtTaTCGTTat

TattgttattagCGtgggTTagggagggTtgCGtaaaagTagTtgg **tggaggagggagag**

atgT **TTtggagtgtgTaa**

ttaTtgTatgtgttgtTaTatatgtgTaatgTTatgTtTTtgag

Unmethylation sequence

agatagtttt tgttttatag ggttgtggtg agaaattaat tagataaggt atgtgaatgt

tattatagta tagtgtttgg tatttagtag gatttatttg atgatagttg ttattgttat

TattgttattagTGtgggTTagggagggTtgTGtaaaagTagTtgg **tggaggagggagag**

atgT **TTtggagtgtgTaa**

ttaTtgTatgtgttgtTaTatatgtgTaatgTTatgTtTTtgagT

met_enrichSHP1F 5`-TGGAGGAGGGAGAGATGGC-3`

SHP1R 5`-AACACATATATACCTTACACACTCCAAA-3`

APPENDIX D

RECIST (Response Evaluation Criteria in Solid Tumors) CRITERIA version 1.1

Measurability of tumor at baseline

1. Measurable tumor lesion

   Must be accurately measured in at least one dimension with a minimum size of;

   - 10 mm by CT scan (CT scan slice thickness no greater than 5 mm)
   - 10 mm caliper measurement by clinical exam (lesions which cannot be accurately measured with calipers should be recorded as non-measurable)
   - 20 mm by chest X-ray

   Malignant lymph nodes: to be considered pathologically enlarged and measurable, a lymph node must be > 15 mm in short axis when assessed by CT scan (CT scan slice thickness recommemded to be no greater than 5 mm). At baseline and in follow-up, only the short axis will be measured and follow.

2. Non-measurable tumor lesion

   All other lesions, including small lesions (longest diameter <10 mm or pathological lymph nodes with > 10 to < 15 mm short axis) as well as truly non-measurable lesions. Lesions considered truly non-measurable include: leptomeningeal disease, ascites, pleural or pericardial effusion, inflammatory breast disease, lymphangitic involvement of skin or lung, abdominal masses/abdominal organomegaly identified by physical exam that is not measurable by reproducible imaging techniques.

Response criteria

1. Evaluation of target lesions

1.1 Complete response (CR)	Disappearance of all target lesions. Any pathological lymph nodes must have reduction in short axis to < 10 mm.
1.2 Partial response (PR)	At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters.
1.3 Progressive disease (PD)	At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study. In

addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm.

1.4 Stable disease (SD) Neither sufficient shrinkage to qualify For PR nor sufficient increase to qualify for PD,taking as reference the smallest sum diameters while on study.

2. Evaluate of non-target lesions

2.1 Complete response (CR) Disappearance of all non-target lesions and normalization of tumor marker level. All lymph nodes must be non-pathological in size (<10 mm short axis)

2.2 Non-CR/Non-PD Persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

2.3 Progressive disease (PD) Uniequivocal progression of existing non-

target lesions.

Table D: Time point response: patients with target (± non-target) disease.

Target lesions	Non-target lesions	New lesions	Overall response
CR	CR	No	CR
CR	Non-CR/non-PD	No	PR
CR	Not evaluated	No	PR
PR	Non-PD or not all evaluated	No	PR
SD	Non-PD or not all evaluated	No	SD
Not all evaluated	Non-PD	No	Inevaluable
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Eisenhauer E.A. et al. New response evaluation criteria in solid tumors revised RECIST guideline (version 1.1) EJC 2009.

ECOG (Eastern Cooperative Oncology Group) performance status

Grade	
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g. light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities, Up and about more than 50% of walking hours
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours
4	Complete disabled. Cannot carry on any selfcare, Total confined to bed or chair
5	Dead

Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982 .

BIOGRAPHY

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2000-2003 Internship of internal medicine division, Rajchaburi Hospital, Thailand

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Honors and award

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Publication

1. Vinayanuwattikun C, Wongsurakiat P. The association between endobronchial intracuff pressure and ventilator-associated pneumonia. Internal medicine journal of Thailand 2006; 22:2s (suppl; abstr21).

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