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Molecular Prognostic Study of Non-Small Cell Lung Cancer using high-throughput tissue microarray and immunohistochemistry

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Abbreviations

ABC	Avidin-biotin complex
ADC	Adenocarcinoma
AP	Apurinic/apyrimidinic
BER	Base excision repair
BSA	Bovine Serum Albumin
CAM	Cell adhesion molecule
DAB	Diaminobenzidine
DSB	Double strand break
H&E	hematoxylin and eosin
HRR	Homologous recombination repair
IHC	Immunohistochemistry
LDH	Lactate dehydrogenase
M	Metastasis status
MMR	Mismatch repair
N	Lymph node status
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NSCLC	Non-small cell lung cancer
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PBS	Phosphate Buffered Saline
PCI	Positive stained cell index
RR	Relative risk
SCC	Squamous cell carcinoma
T	Tumor status
TMA	Tissue microarry

UICC	Union Internationale Contre le Cancer
WHO	World Health Organization

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Zusammenfassung

Einleitung

Lungenkrebs ist die Krebserkrankung, in deren Folge weltweit die meisten Todesfälle auftreten. Während in den letzten Jahren in Europa und den USA die Morbidität und Sterblichkeitsrate von Lungenkrebs bei den Männern abgenommen hat und bei den Frauen stagniert, nimmt sie in den Entwicklungsländern dagegen deutlich zu.

Lungenkrebs wird in Abhängigkeit von der Morphologie und dem klinischen Erscheinungsbild in zwei Hauptgruppen eingeteilt. Dies sind der kleinzellige Lungenkrebs (small cell lung cancer - SCLC) sowie der nicht-kleinzellige Lungenkrebs (non small cell lung cancer - NSCLC), der 80% aller Lungenkrebsfälle umfasst.

Der kleinzellige Lungenkrebs erfasst als sehr heterogene Gruppe eine Reihe verschiedener Tumor-Entitäten. Diese können auf einen gemeinsamen, zellulären Ursprung zurückgeführt werden, unterscheiden sich aber deutlich in ihrem klinischen Verhalten. Aufgrund dieser Heterogenität ist eine Prognose für den einzelnen Patienten schwer zu erstellen.

Die Charakterisierung eines Lungentumors erfolgt derzeit hauptsächlich auf der Basis des TNM-Systems, das morphologische und anatomische Variationen des Tumors genauer erfasst und beschreibt. Eine exakte Prognose ist für den Patienten hiermit aber auch nicht möglich, da Patienten mit demselben NSCLC-Grad und bei gleicher Therapie oft unterschiedlich reagieren.

Daher sind zusätzliche, vor allem biologische Parameter notwendig um die Unterschiede in der Überlebensrate der einzelnen Patienten zu erklären.

Untersuchungen und Studien an NSCLC konzentrieren sich dementsprechend immer mehr auf die Identifikation neuer prognostischer Faktoren, die eine gezielte und individuellere Therapie der Patienten ermöglichen sollen. Aufgrund der Entwicklung der letzten Jahre in der Molekularbiologie hat das Wissen über die Karzinogenese von Lungenkrebs sowie den Mechanismen der Invasion und der Metastasierung stark zugenommen. In diesem Rahmen wurden Faktoren der Proliferation, der Adhäsion und der DNA-Reparatur sowie Regulatoren des Zellwachstums, des Zellzyklus und der Apoptose eingehender untersucht. Hinsichtlich ihrer Qualifikation als prognostische Marker konnten aber vor allem aufgrund konträrer Ergebnisse noch keine abschließenden Aussagen getroffen werden.

Im Rahmen dieser Studie sollten daher mögliche molekulare Prognosefaktoren für den kleinzelligen Lungenkrebs genauer charakterisiert werden. Hierfür wurde die Expression von Rad51 und Ape/Ref1 (DNA-Reparatur), p53 (Zellzyklus), Her2 (Zellwachstum) sowie E-Cadherin und ß-Catenin (Adhäsion) immunhistochemisch in „Gewebe Mircro-Arrays“ von Lungenkrebstumoren untersucht.

Methode

Für die Studie wurden 383 Patienten mit NSCLC mit einem pathologischen Stadium („Stage“) 1 bis 4 untersucht. Alle Patienten waren chinesischer Abstammung. Die chirurgische Entfernung des jeweiligen Tumors wurde zwischen Januar 1994 und Dezember 1997 im „Lung Cancer Research Institute of Guandong Province“, P. R. China, vorgenommen. Die Tumoren wurden anschließend in 10% Formalin fixiert und in Paraffin eingebettet. Die letzten Nachbeobachtungs-Daten („follow up“) der Patienten wurden im Januar 2003 erhoben.

Zum Zeitpunkt der Diagnose betrug der mittlere Zeitraum der Nachbeobachtung der Patienten 34 Monate (4 bis 106 Monate), das mittlere Alter 59 Jahre (22-94 Jahre). 71,3% der Patienten waren männlich. Die Klassifizierung der Tumoren erfolgte gemäß der „International Union Against Cancer’s Tumor-Node-Metastasis (TNM)“, die histologische Einteilung und das „Grading“ wurde

nach den Richtlinien der „World Health Organization“ vorgenommen.

Die histopathologische Untersuchung der Tumoren ergab, dass 192 Adenomkarzinome, 132 Plattenepithel-Karzinome, 44 adenosquamöse Karzinome, 2 großzellige Karzinome, 5 Lungensarkome und 8 wenig verbreitete maligne Neoplasien waren (Karzinoid, Adenoidzystisches Karzinom und Mukoepidermoidkarzinom).

Die postoperative histologische Beurteilung ordnete 110 Tumore dem Stadium 1, 92 Tumore Stadium 2, 124 Tumore Stadium 2a, 37 Tumore Stadium 3b und 21 Tumore dem Stadium 4 zu.

Der immunhistochemische Nachweis der einzelnen Proteine erfolgte auf „Gewebe Micro-Arrays“ (TMA). Diese Methode ist neben ihrem zeitsparenden Charakter vor allem durch eine nahezu optimale Vergleichbarkeit der einzelnen Proben untereinander charakterisiert. Fehlerquellen wie unterschiedliche Konzentrationen der einzelnen Reagenzien, Inkubationszeiten und Temperatur sind hier entsprechend minimiert.

Die gefärbten TMAs wurden unter dem Lichtmikroskop quantitativ ausgewertet. Mindestens 200 Zellen pro Probe wurden gezählt, um den PCI (Anteil positiv gefärbter Kerne) zu ermitteln. Für den Marker-Index wurde ein optimaler Trennpunkt („cut-off“) gewählt, der die beste Einteilung der Patienten in Gruppen mit positiver und negativer Prognose erlaubt. Fälle, die unterhalb des Trennpunktes lagen, wurden als Niedrig-Exprimierer, Fälle, die über dem Trennpunkt lagen, als Hoch-Exprimierer bezeichnet.

Die statistischen Analysen wurden mit der Software SPSS 10.0 durchgeführt. Die Korrelationen der Protein-Expression mit einem gegebenen kategorisierten Parameter wurden mit dem Mann-Whitney U-Test (zwei Kategorien) oder dem Kruskal-Wallis-Test (für mehrere Kategorien) durchgeführt. Die Kaplan-Meyer-Kurven wurden aus den Überlebensdaten erstellt. Der Log-Rank-test wurde für die Analyse der unterschiedlichen Expression der einzelnen Patienten untereinander angewendet. Die multivariate Überlebensanalyse wurde mit dem „Cox Proportional Regression Hazard Model“ durchgeführt.

Ergebnisse

Die Ergebnisse zeigen, dass Rad51, Ape/Ref1, p53 und Ki67 im Kern lokalisiert sind. Für Ape/Ref1 konnte vereinzelt eine zytoplasmatische Anfärbung beobachtet werden. Eine Überexpression konnte für Rad51 in 27,7%, für Ape/Ref1 in 28,7% der Fälle nachgewiesen werden. Die Überexpression von Ape/Ref1 (p=0,028) und p53 (0,009) korrelierte mit der Zelldifferenzierung. Die Expression von Ki67 war in Plattenepithel-Karzinomen und Adenosquamösen Karzinomen signifikant höher als in den Adenokarzinomen (<0,001). Eine signifikante Korrelation konnte zwischen der Expression von Ki67 und dem klinischen Stadium nachgewiesen werden (P=0,009)
Her2, E Cadherin und ß-Catenin wurden hauptsächlich im Zytoplasma und an der Zellmembran nachgewiesen. Die Expression von Her2 korrelierte mit dem Differenzierungsgrad des Tumors (p=0,035), während die E-Cadherin-Expression deutlich mit dem Tumorstatus (p=0,011), dem Status der Lymphknoten (p=0,021), dem klinischen Stadium (p=0,004) und der Tumorzelldifferenzierung (p=0,019) korrelierte. ß-Catenin war dagegen nur mit nur dem Status der Lymphknoten assoziiert (N-Status, p=0,021).

Die Überexpression von p53 war signifikant mit hohen Expressionen von Ki67 (p=0.002), Rad51 (p=0.048) und Her2 (p=0.004) assoziiert. Eine entgegen gesetzte Assoziation wurde zwischen dem Expressionsstatus von Rad51 und Ape/Ref1 (p=0,001) sowie Rad51 und Her2 (p=0,005) beobachtet. Die Korrelation zwischen der E-Cadherin und der ß-Catenin-Expression war ebenfalls signifikant.

Die univariate Überlebensanalyse zeigte, dass eine hohe Expression von nukleärem Ape einen prognostischen Marker darstellt (p=0,040). Die hohe Expression von Rad51 ist mit einer kürzeren Überlebenszeit der Patienten mit Plattenepithel-Karzinom assoziiert (p=0,001). Zusätzlich hat die Expression von p53, Her2 und ß-Catenin eine prognostische Rolle beim Plattenepithel-Karzinom.

Die multivariate Überlebensanalyse zeigt, dass E-Cadherin der einzige unabhängige molekulare prognostische Faktor in dieser Studie war (RR 1.720; 95% CI 1.445-2.046; p<0.001) zusammen mit dem Stadium („Stage“) (p=0,001), dem Status der Lymphknoten (p=0,040), und der Tumorzell-Differenzierung (p=0,001).

Diskussion

Die Proteine Rad51 und Ape/Ref1 nehmen im Rahmen der genomischen Stabilität und der Karzinogenese eine wichtige Funktion ein. Eine veränderte Expression beider Proteine scheint in Krebserkrankungen der Brust, der Prostata und der Bauchspeicheldrüse eine Rolle zu spielen. Die vorliegende Studie zeigt, dass Rad51 eine prognostische Rolle beim Plattenepithel-Karzinom zu spielen scheint, während eine niedrige Expression von Ape/Ref1 einen signifikant ungünstigen Einfluss auf die Überlebensrate der an NSCLC erkrankten Patienten hat.

Ferner konnte diese Arbeit Literaturdaten bestätigen, nach denen das Expressionsniveau von p53, Her2 und ß-Catenin ebenfalls eine prognostische Bedeutung im Rahmen einer NSCLC-Erkrankung besitzen. Eine hohe Proteinexpression von Her2 oder eine niedrige Proteinexpression von ß-Catenin sind ungünstige Prognose-Faktoren für Plattenepithel-Karzinome während die hohe p53-Proteinexpression ebenfalls ungünstig für Adenokarzinome der Lunge ist.

E-Cadherin konnte in dieser Arbeit als einziger unabhängiger prognostischer Faktor identifiziert werden. Der Nachweis einer hohen Proteinexpression von E-Cadherin ist, unabhängig von Zelltyp, in jedem Fall ein ungünstiger Faktor für NSCLC-Erkrankungen.

Die Ergebnisse dieser Arbeit zeigen, dass verschiedene zelluläre Mechanismen wie Signaltransduktion, Zellproliferation oder Zell-Zyklus grundsätzlich an der Entwicklung und Progression von NSCLC-Erkrankungen beteiligt sind.

Die hier untersuchten Proteine könnten in der klinischen Anwendung mit dazu beitragen, neben konventionellen Behandlungsmethoden die Therapie von Lungenkrebs genauer auf den einzelnen Patienten abzustimmen. Darüber hinaus wird es Gegenstand künftiger Forschung sein, Medikamente zu entwickeln, die in den entsprechenden Signalwegen der hier untersuchten Proteine wirken.

1 Introduction

1.1 General aspects of lung cancer

Lung cancer continues to be the most frequent cancer all over the world and is expected to have a major impact on human health throughout the next decades. It accounts for 30% of all cancer deaths with approximately 1 million people worldwide dying of this disease each year [Carney, 2002]. Primarily because of the growing contribution from developing countries, the incidence increases by approximately 0.5% each year [Weir et al, 2003].

Lung cancers are divided into two main groups according to their histology and clinical features, namely small cell lung cancer **(SCLC)** and non-small cell lung cancer **(NSCLC)**, which accounts for approximately 80% of all lung cancers. NSCLC is best conceptualized as a group of heterogeneous clinical entities that share a common cellular origin but have different clinical behaviors, and hence, different prognoses.

Despite enormous progression and improvement in both research and management of lung cancer, the mortality rate of NSCLC has kept unchanged for decades. Therefore, further huge efforts in a variety of research fields should be made to investigate the disease.

1.1.1 Recent progression in the epidemiology of NSCLC

1.1.1.1 Occurrence of NSCLC

Lung cancer was a rare disease at the start of 20th century. At the start of this century, however, it has become one of the world's leading causes of preventable death [Anthony and Jonathan, 2003]. Lung cancer tends to be most common in the developed

countries, in North America and Europe, and less common in developing countries, particularly in Africa and South America. Recently, two notable phenomena have determined the occurrence of lung cancer. The first is the growing incidence of the disease among women most probably as a result from the increased number of smokers among women over the past decades. At present, incidence and death rates from lung cancer in the United States continue to decline in men and have reached a plateau in women [Jema, 2001]. Secondly, the morbidity of the disease tends to subside in developed countries, whereas in developing countries lung cancer is experiencing a rapid increase, possibly due to industrialization and progressive pollution in these countries [Boffetta and Parkin, 1994].

1.1.1.2 Risk factors of NSCLC

Although it appears that the causes of NSCLC are almost exclusively environmental, it is likely that there is substantial individual variation in the susceptibility to respiratory carcinogens. The risk to develop lung cancer can be conceptualized as reflecting the joint consequences of the interrelationship between (1) exposure to etiologic (or protective) agents and (2) the individual susceptibility to these agents.

Smoking (either active or passive), as well as occupational exposures, asbestos, radiation and air pollution have been identified as risk factors of lung cancer. Fruit and vegetable consumption, as well as some micronutrients are considered protective factors. Given this multitude of risk factors, a practical question is their relative contribution to the overall burden of lung cancer. The "population attributable risk" approach takes into account the magnitude of the relative risk that is associated with an exposure along with the likelihood of exposure in the general population. According to these risk estimates, active smoking is responsible for up to 90%, occupational exposures to carcinogens for approximately 9 to 15%, radon causes 10%, and outdoor air pollution accounts for perhaps 1 to 2% of lung cancer cases [Samet and Cohen, 1999]. The precise contribution of nutritional factors remains to be determined, but

dietary factors have been hypothesized to account for approximately 20% (range, 10 to 30%) of the lung cancer burden [Willett, 1995]

1.1.1.3 Distribution of the histological types of NSCLC

Another noteworthy epidemiological aspect is the shift of the distribution of the histological type of lung cancer. In the initial decades of the last century, squamous cell carcinoma was the most frequent type of lung cancer observed among smokers, and small cell carcinoma was the second most frequent. In the late 1970s, the first evidence of a shift towards a predominance of adenocarcinoma was noted [Vincent, RG et al., 1977; Charloux, A et al., 1999] and today adenocarcinoma of the lung is the most common histological type of lung cancer in North America [Travis et al., 1995] and Asia [Hirayama 1981; Kung et al., 1984; Takise et al., 1988; Ko et al., 1997]. By contrast, squamous cell carcinoma remains the most frequent subset in Europe [charloux et al., 1997]. The decline in lung cancer rates in Western countries has been more rapid for squamous cell and small cell carcinomas than for adenocarcinoma, which is just beginning to show a lower incidence rate. Changes in the characteristics of cigarettes and consequently the doses of carcinogens inhaled have been hypothesized to be the underlying basis of this shift in histopathology [Wynder and Muscat, 1995; Hoffmann and Hoffmann, 1997]. An increase in the dose of the potent tobacco-specific nitrosamine NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) has been postulated as one factor leading to the increase in adenocarcinoma [Hecht. 1999]

1.1.2 Diagnosis, histological type and Staging of NSCLC

1.1.2.1 Diagnosis of NSCLC

The diagnosis of NSCLC depends on symptoms, physical examination, radiology (including X-ray examination, CT, etc.) and histopathological findings. The smoking

history provides important clues for the diagnosis as well. The most common symptoms include cough, dyspnoea, chest pain and haemoptysis. Patients with these symptoms should have chest radiography, even CT, to obtain detailed information about a potential pulmonary tumor [Pearlberg et al., 1988]. The most important way to confirm the diagnosis is to establish cytology and histology of the tumor. Sputum cytology, flexible fiberbronchoscopy, percutaneous fine-needle aspiration, mediastinoscopy, video-assisted thoracoscopic surgery, respectively and combined, are helpful techniques for histopathological confirmation [Utz et al., 1993; Dasgupta et al., 1998; Cox et al., 1984]

Since the treatment strategy depends on the histology and stage of the NSCLC and adequate treatment planning begins with a proper diagnosis, clinicians should not only confirm the diagnosis of the disease but also should be aware of the histological type and stage of the tumor [Zarbo et al., 1992].

1.1.2.2 Histological type of NSCLC

NSCLC occurs in multiple histological types as classified by conventional light microscopy. The four major types include squamous cell carcinoma, adenocarcinoma, large cell carcinoma and adenosquamous carcinoma. Together, these four types of lung cancer account for more than 90% of lung cancer cases. Despite extensive research, the mechanisms leading to these different types of lung cancer remain uncertain. Hypotheses have focused on the cells of origin and on the pathways of differentiation of malignant cells [Wynder and Covey. 1987].

The second edition of the World Health Organization (WHO) histological classification of lung tumors was published in 1981. It is the most frequently used histological classification system so far. Common lung neoplasms are classified by evaluating the best-differentiated area of the tumor and graded by the most poorly differentiated region.

Usually, a tumor is graded into three categories: poor; moderate and well differentiation. Histological classification of NSCLC by WHO is shown in Table 1:

Lung tumors	variants
1. Squamous cell carcinoma (epidermoid carcinoma)	Spindle cell (squamous) carcinoma
2. Adenocarcinoma	a. Acinar adenocarcinoma
	b. Papillary adenocarcinoma
	c. Bronchiolo-alveolar carcinoma
	d. Solid carcinoma with mucus formation
3. Large cell carcinoma	a. Giant cell carcinoma
	b. Clear-cell carcinoma
4. Adenosquamous carcinoma	
5. Carcinoid tumor	
6. Bronchial gland carcinoma	a. Adenoid cystic carcinoma
	b. Mucoepidermoid carcinoma
	c. Others
7. Others	

Table 1. Histological classification of NSCLC (WHO, 1981) *NSCLC is divided into 7 subgroups according to morphological features and origins*

1.1.2.3 Staging of NSCLC

Staging of NSCLC, based on the anatomic extent of the disease and described by the TNM staging system (T: primary tumor; N: regional lymph nodes; M: distant metastasis), is an important parameter for determining the clinical course of this disease [Sobin and Wittekind, 1997]. The main goals of TNM staging are to assist in determining appropriate treatment options (surgery versus non-surgical treatment) and in predicting prognosis [Grondin and Liptay, 2002]

The new TNM staging system for NSCLC was revised and unified with the earlier staging system by the American Joint Committee on Cancer and the Union Internationale Contre le Cancer (UICC) in 1997. This revised TNM system can simultaneously yield prognostic information on the tumor and assist in selecting the appropriate therapy [Mountain, 1997]. The summary of the TNM staging system according to UICC (1997) are shown in Table 2 and Table 3:

Category	Definition

T	**Primary tumor**
T_X	Positive cytology
T_1	Diameter of 3 cm or smaller
T_2	Diameter of over 3 cm, tumor in main bronchus but 2 cm or more from carina, invades visceral pleura, partial atelectasis
T_3	Invades chest wall, diaphragm, pericardium, mediastinal pleura, tumor in main bronchus to less than 2 cm from carina, total atelectasis
T_4	Invades mediastinum, heart, great vessels, carina, trachea, esophagus, vertebra; separate nodules in same lobe, malignant effusion
N	**Regional lymph nodes**
N_0	No lymph node involved
N_1	Ipsilateral peribronchial, ipsilateral hilar
N_2	Ipsilateral mediastinal, subcarinal
N_3	Contralateral mediastinal or hilar, scalene or supraclavicular
M	**Distant metastasis**
M_0	No remoter metastasis
M_1	Distant metastases, includes separate nodule in different lobe

Table 2. Definition of T, N and M for NSCLC by UICC (1997) *the status of primary tumor, lymph node and distant metastasis are categorized into subsets*

Stage	T status	N status	M status
Occult carcinoma	T_X	N_0	M_0
Stage 0	T in situ	N_0	M_0
Stage Ⅰ$_A$	T_1	N_0	M_0
Stage Ⅰ$_B$	T_2	N_0	M_0
Stage Ⅱ$_A$	T_1	N_1	M_0
Stage Ⅱ$_B$	T_2	N_1	M_0
	T_3	N_0	M_0
Stage Ⅲ$_A$	T_{1-2}	N_2	M_0
	T_3	N_{1-2}	M_0
Stage Ⅲ$_B$	Any T	N_3	M_0
	T_4	Any N	M_0
Stage Ⅳ	Any T	Any N	M_1

Table 3. TNM staging system of NSCLC by UICC (1997) ***NSCLC is divided into subgroups according to status of T, N and M***

1.1.3 Treatment of NSCLC

The success rate of current treatment regimes for lung cancer is not encouraging. The expected 5-year survival rate for all patients diagnosed with NSCLC is 15%, compared with 61% for colon cancer, 86% for breast cancer, and 96% for prostate cancer [Jemal et al., 2002]. Progress in treatment has been slow. The current overall 5-year survival rate of 15% is only slightly better than the 8% survival rate of the early 1960s.

Nowadays, the standard treatment for NSCLC is according to the guidelines from several authoritative organizations, such as the Association for Lung Cancer Study. All of these guidelines for management of NSCLC are based on TNM staging. Surgery remains the treatment of choice for early stage NSCLC and results in promising long term survival for these patients. In contrast, chemo- and radio-therapy, respectively or combined, are the most effective approaches for most of the advanced cases of NSCLC. With the increasing understanding and progression of molecular mechanisms of NSCLC, gene therapy and targeted therapy provide new hope for the treatment of advanced and inoperable NSCLC. Recent evidence demonstrates that a combination of surgery and chemo- or radio-therapy could improve the survival and life quality of NSCLC patients [Stewart and Pignon, 1995]. However, the exact effectiveness of these approaches and their combination are still unclear.

For early stage NSCLC, surgery provides the chance of complete cure, but the type of surgery to be applied has been discussed for several decades. Many investigations have shown that patients who underwent complete tumor resection and systematic mediastinum lymph node dissection have a longer median survival time than patients who underwent conservative resection [Wu et al., 2002]. For advanced NSCLC, chemotherapy is known to improve median survival time when compared to the best supportive care alone. Cisplatin based chemotherapy regimens, particularly combined with third generation chemotherapy agents (such as Taxol, Gemcitabine), have a favorable impact on survival [Hosoe et al., 2003].

1.2 Prognostic factors of NSCLC

The treatment of lung cancer requires an excellent understanding of the prognosis of the disease for the individual patient. Therefore, a substantial amount of clinical and basic research has focused on the identification of prognostic factors over past decades. Early investigations examined clinical characteristics of the tumor and the patient, such as the extent of the disease and weight loss, respectively. Subsequently, a number of clinical laboratory tests, such as serum lactate dehydrogenase (LDH) levels, were identified as being relevant. Recently, most investigations concentrate on new parameters arising from an increased understanding of the cellular and molecular biology of lung cancer [Michael et al., 2002].

1.2.1 Host related factors

Many studies have addressed patient characteristics as predictors of survival. These factors are not generally considered to be important for clinical decision-making, especially for early stage NSCLC. However, weight loss and performance status are the most predictive indicators for median survival time after undergoing systemic chemotherapy for advanced and terminal NSCLC [Albain et al., 1991].

Other host related factors including sex, age and smoking habit, as well as quality of life, symptoms and depressed mood have been investigated over the past decades. However, the prognostic role of these factors remains controversial and unclear [Buccheri et al., 1994].

1.2.2 Tumor related factors

The TNM staging system has consistently proven to be the most powerful prognostic tool for predicting overall survival of NSCLC patients [Mountain et al., 1997]. Additional tumor related prognostic factors include other elements of the anatomic extent of the disease and conventional histological parameters, which are clinically useful in estimating prognosis.

With regard to histological parameters, the prognostic significance of the tumor cell type (*e.g.,* large cell undifferentiated, adenocarcinoma, or squamous cell) has been studied extensively. Many of these studies have identified adenocarcinoma as independent prognosticator with negative impact on patient survival [Buccheri et al., 1994], whereas other studies of comparable design could not confirm these findings [Fontanini et al., 1997]. Bronchoalveolar cancer and carcinoid tumors constitute notable exceptions, as each is considered to be a distinct clinical entity with natural histories that differ from the more common NSCLC tumor types [Clayton, 1988; Perkins et al., 1997]. In addition, histological differentiation is widely accepted as prognostic factor for NSCLC. Patients with poorly differentiated NSCLC are thought to have shorter survival time than those with well differentiated NSCLC [Buccheri et al., 1994].

Many other tumor factors (*e.g.,* neuron specific enolase, etc.) have been reported to have independent prognostic significance in NSCLC. However, these factors are not typically assessed in routine clinical practice, because their prognostic value remains uncertain [Buccheri et al., 1994].

1.2.3 Prognostic markers for NSCLC

The TNM stage emphasizes on morphologic and anatomic variations of NSCLC. Although the TNM stage is the most significant prognostic parameter, it cannot predict the prognosis for the individual NSCLC patient exactly. Patients even with the same

stage of NSCLC having received the identical therapy may manifest a different outcome. Therefore, additional biological parameters are required for explaining this variability of survival.

Based on new developments in molecular biology, our knowledge about lung carcinogenesis and mechanisms for invasion and metastasis has expanded. Hence molecular and cellular prognostic factors of lung cancer have been extensively studied recently. These factors include: (1) Host-related markers, (*e.g.,* CYPIA-1 gene polymorphism) [Goto et al., 1994]*;* (2) Markers of tumor proliferation, (*e.g.,* Ki-67, telomerase, PCNA) [Michael et al., 2002]*;* (3) Markers of cellular adhesion, invasion, metastasis and angiogenesis, (*e.g.,* E-Cadherin, ß-catenin, MMPs, CD24, CD44, nm23, CD82, angiogenesis factor □, MMP) [Michael et al., 2002]; (4) Molecular biological markers, including regulators of cellular growth (*e.g.,* HER2, Ras, Rb, EGFR, VEGF, VEGF receptors), regulators of the metastatic cascade and regulators of cell cycle and apoptosis (*e.g.,* p53, cyclin D-1, cyclin E, bcl-2, p27 ,p16 ,p21, cyclin A, cyclin E, APC and cdk2) [Michael et al., 2002]; and (5) Others, such as DNA repair factors [Caporaso et al., 2003].

Up to now, none of these markers has gained sufficient evidence to serve as an exclusive discriminator for distinguishing different outcomes and guiding individual treatment [Michael et al., 2002].

1.2.3.1 p53 and the prognosis of NSCLC

In many types of human cancers, including NSCLC, the p53 tumor suppressor is completely inactivated when one copy of the gene is mutated and the remaining allele is subsequently deleted [Harris, 1996]. P53 protein is thought to act as a negative regulator of cellular proliferation or as an inducer of apoptosis through the transactivation of genes, including p21, BAX, and GADD45. Misssense mutations in p53 gene usually but not always prolongs the half-life of the protein from minutes to

hours and result in nuclear accumulation of the p53 protein, which can be detected by immunohistochemistry (IHC) [Harris, 1996].

The p53 gene is the most extensively studied prognostic marker in NSCLC with more than 100 reports on this topic [Mitsudomi et al., 2000]. However, there is a great controversy as to whether deregulation of p53 functions negatively affects survival of NSCLC patients. Recently, Tan and coworkers reported that over-expression of p53 is a favorable independent prognostic factor for NSCLC [Tan et al., 2003]. On the other hand, a Meta-analysis indicated that the overall incidence of p53 alterations in NSCLC was 48.2% and that p53 alteration was a significant marker of poor prognosis in patients with pulmonary adenocarcinoma [Mitsudomi, 2000].

1.2.3.2 Ki67 and the prognosis of NSCLC

Although expression of the Ki-67 polypeptide is vital for cell proliferation, little is known about the exact function of this protein [Schluter et al., 1993]. The similarity of part of Ki67 with known cell cycle regulators, e.g. DUN1 and RAD53, might imply a similar role for Ki67 [Li et al., 2004; Lee et al 2003; Hofmann and Bucher, 1995]. Based on its localization to extranucleolar sites containing centromeric and satellite DNA, Bridger and co-workers suggested a role for Ki-67 in organizing DNA during early G_1 [Bridger et al, 1998]. The ability of this protein to interact with other proteins, as well as RNA and DNA might point towards an architectural or structural role for Ki67 protein within the nucleolus. They also suggested that Ki67 is an essential factor in the synthesis of ribosomes during cell division [Bridger et al., 1998].

It is clear that the Ki67 expression is of prognostic value for many types of malignant tumors (e. g. breast cancer). For NSCLC, the results have been contradictory, with some studies showing Ki67 to have a strong prognostic value and others little or none. Two studies report that Ki67 expression is significantly correlated with the clinical

outcomes of NSCLC patients using immunohistochemical analysis [Hommura et al, 2000; Haga et al., 2003]. However, the 10 years follow up study by Carbognani and colleagues did not show any relationship between Ki67 expression and survival of NSCLC patients [Carbognani et al., 2002].

1.2.3.3 HER2 and the prognosis of NSCLC

HER2 is localized in normal human cells as a singular copy gene on the long arm of chromosome 17. This gene codes for a 185-kDa receptor-type tyrosine protein kinase (p185 neu or c-erbB-2) similar to EGFR. HER2 is necessary for the regulation of normal cell growth and differentiation, and is associated with multiple signal transduction pathways [Hung and Lau, 1999]. However, amplification of the HER2 gene leads to overexpression of the receptor and is implicated in the development of many types of tumors [Zhou and Hung, 2003]

The HER2 protein is expressed in 20-30% of NSCLC and particularly in adenocarcinoma [Tateishi et al., 1991]. Numerous studies have suggested that HER2 expression is associated with advanced or metastatic disease and poor prognosis, whereas others did not [Giatromanolaki et al., 1996] .Up to date, more than 30 studies have focused on the prognostic role of HER2 expression and NSCLC survival, but the results remain conflicting [Meert et al., 2003].

1.2.3.4 E-cadherin and β- catenin and the prognosis of NSCLC

The cadherins are considered the most important of four groups of cell adhesion molecules (CAMs) involved in cell-cell and cell-matrix adhesion. Cadherins are single transmemberane proteins that mediate cell-cell adhesion in a strictly Ca^{2+} dependent manner, and have been divided into more than 10 subgroups, depending on their tissue distribution. Among the subgroups, E-cadherin is the key component for adherence junctions between epithelial cells [Shapiro., et al., 1995].

For the extracellular E-cadherin-mediated cell-cell adhesion to function, it requires a complex series of interactions between E-cadherin and a set of cytoplasmic molecules named catenins. The catenins bind the intracellular domain of the cadherin to the actin cytoskeleton [Tucker and Pignatelli, 2000]. This linkage between the transmembrane E-cadherin and actin filaments of the cytoskeleton is necessary for strong cell-cell adhesion. Among the subgroups of the catenin family, ß-catenin binds directly to the cytoplasmic tail of E-cadherin in a mutually exclusive manner. So the E-cadherin and ß-catenin complex plays a major role in maintaining the intercellular junctions in epithelial tissues [Bremnes et al., 2002a]. Recent evidence proposed E-cadherin as an invasion suppressor molecule in carcinoma cells and as a growth suppressor inducing cell cycle arrest via upregulation of CDK inhibitor p27 [Hirohashi, 1998].

Recently, more than 10 studies have demonstrated that aberrant expressions of E-cadherin and ß-catenin are associated with clinicopathological features (e.g., differentiation, stage and metastasis) in NSCLC [Bremnes et al., 2002a]. Univariate survival analysis suggests that reduced expression of either E-cadherin or ß-catenin is associated with unfavorable prognosis in several human malignancies. Moreover, aberrant expression of E-cadherin and ß-catenin were identified as independent prognostic factors for survival in multivariate analyses [Bremnes et al., 2002b]. By contrast, studies of Lee and Ramasami did not show a correlation of reduced E-cadherin and the prognosis of NSCLC [Ramasami et al., 2000; Lee et al., 2002].

1.3 DNA repair and the prognosis of NSCLC

Recent evidence suggests that two systems are essential in humans for genome integrity, DNA repair and apoptosis. Cells that are defective in DNA repair tend to accumulate excess DNA damage. Cells defective in apoptosis tend to survive with excess DNA damage and thus allow DNA replication past DNA damages, causing

mutations leading to carcinogenesis or resistance of tumor cells against chemo- and radio-therapy. Consequently, DNA repair capability plays a dramatic role in the progression of cancer [Henning and Stürzbecher, 2003].

A large body of research revealed the existence of five major DNA repair pathways in human cells: (1) base excision repair (BER); (2) nucleotide excision repair (NER); (3) mismatch repair (MMR); (4) non-homologous end joining (NHEJ); (5) homologous recombination repair (HRR). In each of these DNA repair pathways, key proteins occur with dual functions in DNA damage sensing/repair and apoptosis. It has recently become apparent that key proteins which contribute to cellular survival by acting in DNA repair become executioners in the face of excess DNA damage. Proteins with these dual roles occur in: (1) BER (Ref-1/Ape, poly(ADP-ribose) polymerase-1 (PARP-1) and p53); (2) NER (XPB, XPD, p53 and p33(ING1b)); (3) MMR (MSH2, MSH6, MLH1 and PMS2); (4) NHEJ (the catalytic subunit of DNA-PK); (5) HRR (Rad51, BRCA1, ATM, ATR, WRN, BLM, Tip60 and p53). For a number of these dual-role proteins, germ line mutations or abnormal expression of these proteins cause them to be defective and predispose individuals to cancer. On the other hand, if these proteins present excessive function of DNA repair in tumor cells, these cells might become resistant to therapeutic agents [Bernstein et al., 2002].

NSCLC is frequently resistant to chemotherapy and radiology, and this resistance has been associated with elevated DNA repair capability in tumor tissue [Bosken et al., 2002]. So, it is rational that NSCLC patients with elevated DNA repair capability may have an unfavorable outcome compared to those with normal capability.

1.3.1 Homologous recombination factor Rad51

Homologous recombination is one of the mechanisms involved in the repair of the double strand breaks (DSB). key-factor catalysing these lesions is the product of the Rad51 gene. Induced disruption of the Rad51 gene in chicken cells leads to cell death

accompanied by the accumulation of DNA double-strand breaks [Sonoda et al., 1998:]. In addition, Rad51 protein interacts with a variety of tumor suppressor proteins such as p53 [Stürzbecher et al., 1996], BRACA1 and BRACA2 [Sharan et al., 1997]. Additionally, high recombination frequency along with elevated expression of Rad51 were found in sporadically arisen immortal human cell lines compared to mortal cells [Xia et al, 1997]. High levels of Rad51 are present in tumour cell nuclei of pancreatic adenocarcinoma specimens [Maacke et al., 2000]. Furthermore, Rad51 over-expression correlates with histological grading of the tumour in invasive ductal mammary carcinoma [Maacke et al., 2000]. While elevated expression of Rad51 enhances radioresistance of human tumour cells [Yanagisawa et al., 1998; Vispé et al., 1998]. Ohnishi and colleagues found that treatment of monolayer cultures of tumour cells with Rad51 specific anti-sense oligonucleotides renders them radiosensitive [Ohnishi et al., 1998]. Unpublished data from our laboratory demonstrate that over-expression of Rad51 protein in an experimental tumour cell system renders cells resistant against cytotoxic drugs like calicheamicin, Actinomycin, Staurosporin and Cisplatin. All of this data imply that Rad51 is not only involved in the progress of carcinogenesis but might also be highly significant in clinical practice. Up to date, little is known about relation between expression of Rad51 and prognosis of NSCLC.

1.3.2 Base excision factor Ape/Ref-1

Ape/Ref-1, originally named apurinic/apyrimidinic (AP) endonuclease (Ape-1) and HAP-1 (Human Ape-1), is a multifunctional protein that has an impact on a wide variety of important cellular functions, including oxidative signaling, transcription factor regulation, cell cycle control and cancer [Evans, 2000]. It acts on mutagenic AP sites in DNA as a major member of the DNA base excision repair (BER) pathway [Barzilay and Hickson. 1995]. Simple glycosylases excise damaged bases in the DNA, resulting in apurinic/apyrimidinic (AP) sites that are subsequently incised 5' to the AP site by Ape/Ref-1, allowing repair to be completed by deoxyribose phosphatase activity to

remove the deoxyribose phosphate termini, followed by insertion of the correct base and ligation. Additionally, Ape/Ref-1 is able to repair the 3'-phosphate and phosphoglycolate lesions generated in single strand breaks by ionizing radiation and bleomycin [Hansen et al., 1998]. Moreover, Ape/Ref-1 stimulates the DNA binding activity of transcription factors through a redox mechanism and thus represents a novel component of signal transduction processes that regulate eukaryotic gene expression [Lando, et al., 2000]. Ape/Ref-1 has also been shown to be closely linked to apoptosis [Robertson, et al., 1997], and altered levels of Ape/Ref-1 have been found in some cancers [Xu, et al., 1997; Moor,D.H. et al., 2000; Kelley,M.R.et al., 2001]. Recently, Robertson and colleagues found that elevated expression of Ape/Ref-1 in some cell lines results in resistance to certain therapeutic agents, and they hypothesized that elevated Ape/Ref-1 levels in cancer may be related to therapy resistance and may serve as a diagnostic marker for refractory disease [Robertson, et al., 2001].

With regard to the prognostic role of Ape/Ref-1 in cancer, Puglisi and coworkers immunohistochemically stained 133 stage□-□ breast carcinoma specimens with an Ape/Ref-1 antibody and found that the small group of women whose tumors showed mixed nuclear and cytoplasmic Ape/Ref-1 localization experienced a significantly poorer survival. Cox proportional hazard model analysis identified Ape/Ref-1 as an independent prognostic factor [Puglisi et al., 2002]. As for NSCLC, Kakolyris and colleagues found that high level of Ape/Ref-1 nuclear expression is a favorable prognostic factor in NSCLC, patients with high level of Ape/Ref-1 nuclear expression have better outcome than those with low level of expression [Kakolyris et al., 1999].

1.4 Aim of the study

This study was undertaken to evaluate molecular prognostic factors of NSCLC. Three hundred and eighty-three patients with NSCLC and their corresponding archived formalin fixed and paraffin embedded specimens were recruited. The specimens were used for constructing tissue microarrays. The specific aims of the present study were as follows:

- To establish the methods and procedures for immunohistochemically detecting Rad51 and Ape/Ref-1 in a tissue microarray constructed from specimens from NSCLC
- To explore the expression patterns of Rad51 and Ape/Ref-1 proteins in NSCLC tumors and to investigate the relationship between their expression and clinicopathological features.
- To evaluate the prognostic role of Rad51 and Ape/Ref-1 in NSCLC by univariate and multivariate survival analysis.
- To correlate the expression of Rad51 and Ape/Ref-1 with other most often reported markers, including p53, Ki67, HER2, as well as E-cadherin and ß-catenin.
- To give further evidence on the prognostic role of p53, Ki67, HER2, as well as E-cadherin and ß-catenin in NSCLC.
- To experience and evaluate the application of the newly invented tissue microarray technique in a molecular prognostic study of cancer.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals and reagents were used in analytic degree of purity

2.1.1.1 Chemicals and biochemicals

• Avidin/ Biotin Blocking Kit	Vector Laboratories, Burlingame, USA
• DAB Substrate Kit	Vector Laboratories, Burlingame, USA
• Vectastain Elite ABC-Peroxidase-Kit	Vector Laboratories, Burlingame, USA
• Faramount mounting medium	Dako, Carpinteria, USA
• Mayer's haematoxylin	Merck, Darmstadt, Germany
• Citric acid monohydrate	Sigma, Steinheim, Germany
• Triton X-100	Sigma, Steinheim, Germany
• Albumin bovine	Sigma, Steinheim, Germany
• Hydrochloric acid	Merck, Darmstadt, Germany
• Sodium phosphate dibasic	Merck, Darmstadt, Germany
• Sodium phosphate monobasic	Merck, Darmstadt, Germany
• Sodium chlorate	Merck, Darmstadt, Germany
• Hydrogen Peroxide,30%	Sigma, Steinheim, Germany
• Xylene	Schuchardt, Hohenbrun. Germany
• Ethanol	Apotheke University Luebeck

2.1.1.2 Antibodies and characteristics of these antibodies

- **Anti-Rad51 (clone 1G8)** *[Buchhop et al., 1996]*

  The antibody used for the detection of Rad51 expression is the mouse monoclonal MAB 1G8. This antibody has been described previously (Buchhop et al., 1996; Maacke et al., 2000). It specifically recognizes Rad51 protein in paraffin embedded tissues. Intense staining was highly specific for Rad51 protein, since pre-incubation of the anti-Rad51 antibody with a peptide corresponding to the epitope recognized by 1G8 completely blocked visualization of the Rad51 protein (Maacke H et al., 2000). In addition, databank search revealed that the 1G8 epitope is not present on other known proteins, including human Rad51 paralogs.

- **Anti-Ape/Ref-1(clone** 13B8E5C2**)** *Kindly presented by Ph.D. Mark R. Kellley from HB Wells Center for Pediatric Research Herman, Indianapolis, U. S. A.*

  Clone 13B8E5C2 monoclonal is specific for Ape/Ref-1, and can be used in immunohistochemical detection of a variety of paraffin embedded human tissues.

- **Anti-p53 (Ab-6, clone** DO-1**)** *Oncogene Research Products, Cambridge*

  Clone DO-1 is a mouse monoclonal antibody generated by immunizing BALB/c mice with recombinant human wild type p53 protein and fusing splenocytes with mouse myeloma. Epitope: amino acid residues 21-25 of human p53. It reacts with both mutant and wild type p53.

- **Anti-Ki-67 (Ab1, clone** k-3**)** *Oncogene Research Products, Cambridge*

  Clone K-3 is a mouse monoclonal antibody generated by immunizing mice with recombination Ki-67 protein corresponding to the C-terminal 340 amino acids of human Ki67. It has been being broadly used in immunohistochemical detection of a variety of paraffin embedded human tissues.

- **Anti-HER2 (code** No. A 0485**)** *Dako, Denmark*

  Rabbit anti-human c-erbB-2 oncoprotein is an affinity-isolated antibody purified by using an immobilized c-erbB-2 oncoprotein peptide. The antibody reacts with the c-erbB-2 oncoprotein when tested on formalin fixed, paraffin embedded tissues.

- **Anti-E-cadherin (clone 4A2C7)** *Zymed Laboratories, South San Francisco, USA*

  Clone 4A2C7 ~~is~~ monoclonal antibody is specific for E-cadherin but does show some minor cross reactivity with P- cadherin.

- **Anti-ß-catenin (clone CAT-5H10)** *Zymed Laboratories, South San Francisco, USA*

Clone CAT-5H10 is a monoclonal mouse antibody. It is specific for 92 kDA ß-catenin protein and does not cross react with other subgroups of catenin.

- **Biotinylated secondary anti-mouse or anti-rabbit IgG antibody** *Vector Laboratories, Burlingame, USA*

2.1.1.3 Solution and buffer

- *ABC Reagent:*

The ABC working solution was prepared according to manufacturer's instructions (from Vectastain Elite ABC-Kit: Vector Laboratories, Inc., Burlingame, CA). Briefly, two drops of „Regent A" and two drops of „Regent B" were added into 5 ml PBS and incubated at room temperature for 30 minutes before use. This solution can be stored at 2-8 °C for 3 days.

- *DAB (3,3'- diaminobenzidine) Reagent:*

The working substrate solution was prepared according to manufacturer's instructions (from DAB Substrate Kit: Vector Laboratories, Inc., Burlingame, CA) before use. Firstly, 2 drops of Buffer Stock Solution were added to 5.0 ml of distilled water and mixed well; and then 4 drops of Stock Solution were added and mixed well; finally, 2 drops of the Hydrogen Peroxide Solution were added and mixed well. Afterwards, the tissue sections were incubated with this substrate solution at room temperature until suitable staining develops

- *Sodium Citrate Buffer: 10 mM, pH 6,0:*

To prepare 1 liter, add 2.94 g sodium citrate to 1 liter dH_2O. Adjust pH to 6.0.

- *Alcoholic-hydrochloric acid solution for differentiation of haematoxylin:*

Three ml hydrochloric acid was added to 97 ml of 100% ethanol to prepare the solution for differentiation of over-stained tissues by haematoxylin. This solution can be stored and used for two to three weeks.

- *10X Phosphate Buffered Saline (PBS)*:

To prepare 1 liter of 10X PBS, use 82.33 g Na_2HPO_4, 23.45 g $NaH_2PO_4 \cdot H_2O$ and 40 g NaCl. Adjust pH to 7.4.

- *Triton X-100:, 0,2%:*

Prepare stock of 20% Triton in PBS. Rotate tube overnight to dissolve. Dilute to 0.2% in PBS. .

- *Hydrogen Peroxide: 3%:*

To prepare, add 10 ml 30% H_2O_2 to 90 ml PBS.

- *5% Bovine Serum Albumin (BSA):*

For preparation of 5% BSA, 5 gram albumin bovine was dissolved into PBS, and adjusted to the final volume to 100 ml.

2.1.2 Instruments and equipments

- Adhesive coated slides: *Instrumedics, Inc. Hackensack, USA*
- Microtome: *Vibratome 3000 Plus Automated, CE*
- Pressure cooker
- Humidity chamber
- Wash bottles

- Absorbent wipes
- Coverslips
- Light microscope: *Olympus, BX40*
- Tissue arrayer (MTA-1): *Beecher Instruments, Silver Spring, MD*.

  This first generation manual arrayer is an affordable benchtop unit for laboratories that occasionally construct tissue arrays (Figure 1). The manual arrayer allows for relatively easy construction of tissue microarrays with several hundred specimens (typical size of an array is up to 500 cases).

Figure 1 *MTA-1 manual tissue arrayer.*

2.1.3 Patients and samples

This study was an international cooperation project between Lung Cancer Research Institute of Guangdong province, China and the University of Luebeck, Germany. The protocol was approved by the Ethical Committee of Lung Cancer Research Institute, Guangdong province, P. R. China and the Ethical Committee of University of Luebeck, Germany (Aktenzeichen 03-153).

We used primary tumor tissue samples from patients diagnosed with NSCLC pathological stage I to IV at the Lung Cancer Research Institute of Guangdong province, P.R. China between January 1994 and December 1997. All patients were consecutive Chinese patients with NSCLC and underwent surgery at the department of surgery of

this institute.

Excluding criteria were as follows: (1) Patients who had undergone chemotherapy or radiotherapy prior to surgery; (2) Patients who died within 3 months after surgery; (3) Patients whose cause of death remained unknown.

Ultimately, 383 patients and their corresponding resected tumor specimens were recruited in this study. All surgically resected tumor specimens were fixed with 10% formalin and embedded in paraffin.

All of the patients have been followed for at least five years, and the last follow-up was carried out in January of 2003.

These patients had a median follow-up of 34 months (range from 4 to 106 months). All 383 patients with NSCLC underwent thoracotomy. All detailed information about demography, clinical manifestation and histopathology of the patients was collected retrospectively.

The tumors were staged according to the International Union Against Cancer's tumor-node-metastasis (TNM) classification [Mountain, 1997] and histologically subtyped and graded according to the World Health Organization guidelines [Sobin, 1982]. Information about the staging and the cell type of tumors included in this study was collected from the formal pathological record of the Department of Pathology, Lung Cancer Research Institute of Guangdong province, P.R. China.

Demographic, clinico-pathological and follow-up data are demonstrated in the results section.

2.2 Methods

2.2.1 Construction of NSCLC tissue microarry (TMA)

Generally, the array construction involves making holes in the recipient TMA block and transferring tissue samples from donor tissues to these holes using tissue arrayer. The recipient TMA block is a paraffin block which area is 45 x 25 mm. There are two punches in the tissue arrayer, one is for making holes in the recipient TMA block, and another for punching, transferring and depositing the samples (Figure 2). Usually, a cylindrical core biopsy in 0.6 mm diameter from the donor blocks is punched, and then deposited into the hole in 0.8 mm diameter in the recipient block. This process is repeated to array hundreds of tissue specimens with 0.1mm spacing in the array block. Figure 3 illustrates the process of punching, transferring and depositing of samples in the construction of the tissue microarray.

Figure 2. Tissue arrayer and its working principle. *Adjusting front-back alignment of a punch with the setscrews (3 and 4) located in front of the arrayer. Samples from the donor block are aspirated and arrayed into a new recipient paraffin block (5). 1 is a punch for making a hole in the recipient block, and 2 is a punch for aspirating, transferring and depositing samples.*

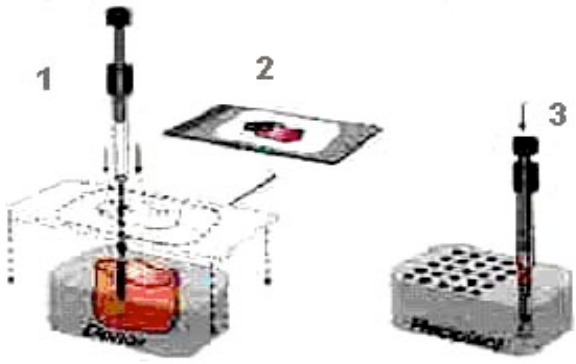


Figure 3. Aspirating, transferring and depositing of sample during construction of TMAs. *The donor block (1) is manually positioned for sampling based on the corresponding HE-stained section on a slide (2). The region of interest for punching in each tumor is carefully selected from the H&E stained slide. A cylindrical core tissue biopsiy (diameter 0.6 mm, height 3-4 mm) is punched from individual donor blocks and arrayed into a new recipient paraffin block (3). (see: http://www.yalepath.org/DEPT/index.htm)*

In the present study, tissue microarrays containing 383 NSCLC specimens were constructed using the MTA-1 manual tissue arrayer. All these archival formalin-fixed paraffin-embedded tumor blocks (donor blocks) were representative histologically and at least 3 mm thick. In order to sample representative regions of the tumor, a fresh section was cut from each donor block and stained with hematoxylin and eosin (H&E). Then this slide was used to guide the sampling from morphologically representative regions of the tissues. In view of the heterogeneity of the tumors, three representative regions from one individual donor sample were sampled and arrayed into the same TMA block.

The whole procedure and instruments used in the construction of tissue microarrays are illustrated in Figure 4.

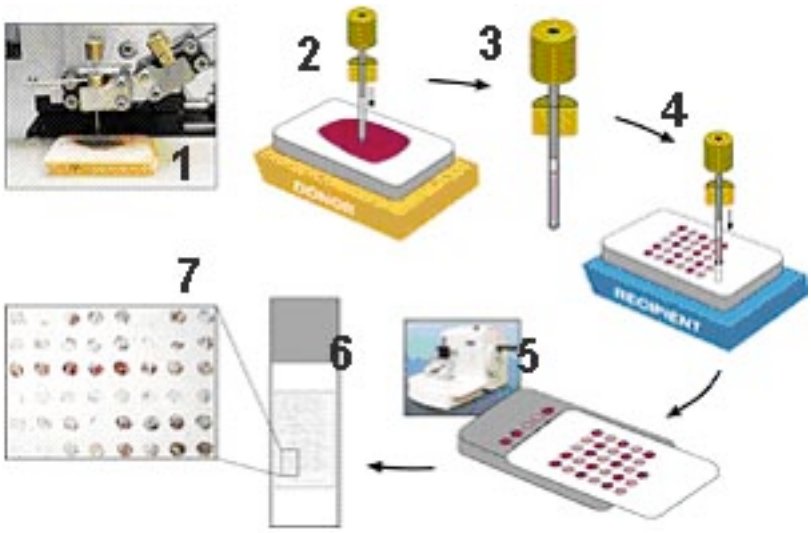


Figure 4. Whole procedure and instruments used in TMA construction. *1:tissue arrayer; 2:puncturing of donor sample; 3:transferring of cylindrical core sample; 4:cylindrical core sample is deposited into recipient block; 5:recipient block is cut into sections by microtome; 6: section cut from recipient block is mounted on slide; 7:magnified picture of TMA..*
(see: http://www.yalepath.org/DEPT/index.htm)

Ultimately, three TMA slides, containing a total of 383 NSCLC tissues and 1 normal lung tissue, as well as three benign diseases of lung, were constructed.

Since the morphology of the tissues may change as more sections were cut, the first section and every 50th section of the TMA blocks were stained with H&E and monitored for morphology and representative of the specimens (Figure 5)

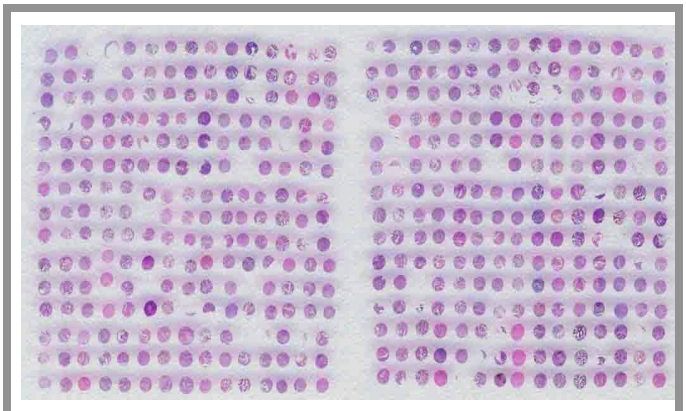


Figure 5. Image of TMA of NSCLC stained with hematoxlin-eosin. *Loss of spots may be due to failure of the section to adhere to the slide or misalignment of the core in the block.*

2.2.3 TMA Slide preparation

Tissue sections (5 μm thick) were cut from paraffin-embedded tumor tissue microarray blocks on a microtome and mounted from warm water (40□) onto adhesive microscope slides. Adhesion of the section to the slide is essential to prevent tissue loss during subsequent incubations and washes. The advantage of using glue coated slides is that orientation of the tissue array sections on the microscope slides could be precisely controlled. Finally, more than 200 sections can be cut from one tumor tissue microarray block. Sections were allowed to dry overnight at 40□ and were then stored in sealed box before use.

2.2.4 Immunohistochemistry (ICH)

In this study, an avidin-biotin complex (ABC) immunoperoxidase technique was used to immunohistochemically detect the expression of various markers.

2.2.4.1 Principle and advantage of ABC method

ABC technique, as a unique immunoperoxidase procedure for localizing a variety of histologically significant antigens and other markers, was originally introduced by Su-Ming Hsu and his associates [Hsu et al., 1981a]. This patented procedure employs biotinylated antibody and a preformed Avidin-Biotinylated enzyme Complex and has been termed the ABC technique.

Because avidin has such an extraordinarily high affinity for biotin (over one million times higher than antibodies for most antigens), the binding of avidin to biotin is essentially irreversible. In addition, avidin has four binding sites for biotin, and most proteins including enzymes can be conjugated with several molecules of biotin. These properties allow macromolecular complexes (ABC complex) to be formed between avidin and

biotinylated enzymes. [Hsu et al., 1981b].

The high sensitivity reported for this technique likely resides in either the form or the number of active enzyme molecules associated with the complex and the rapid, irreversible interaction of the complex with the biotinylated antibody. The ABC method is by far the most sensitive and widely used technique for immunhistochemical staining.

The procedure of ABC technique is as follows: The first step is to incubate the section with primary antiserum raised against the antigen of interest, such as rabbit antibody to a tumor-associated antigen. Next, a biotin-labeled secondary antibody is added, which in this example would be biotinylated anti-rabbit IgG. This introduces many biotins into the section at the location of the primary antibody. The avidin-biotinylated enzyme complex (ABC) is then added and binds to the biotinylated secondary antibody. In the last step of the procedure, the tissue antigen is localized by incubation with a substrate for the enzyme (Figure 6.).

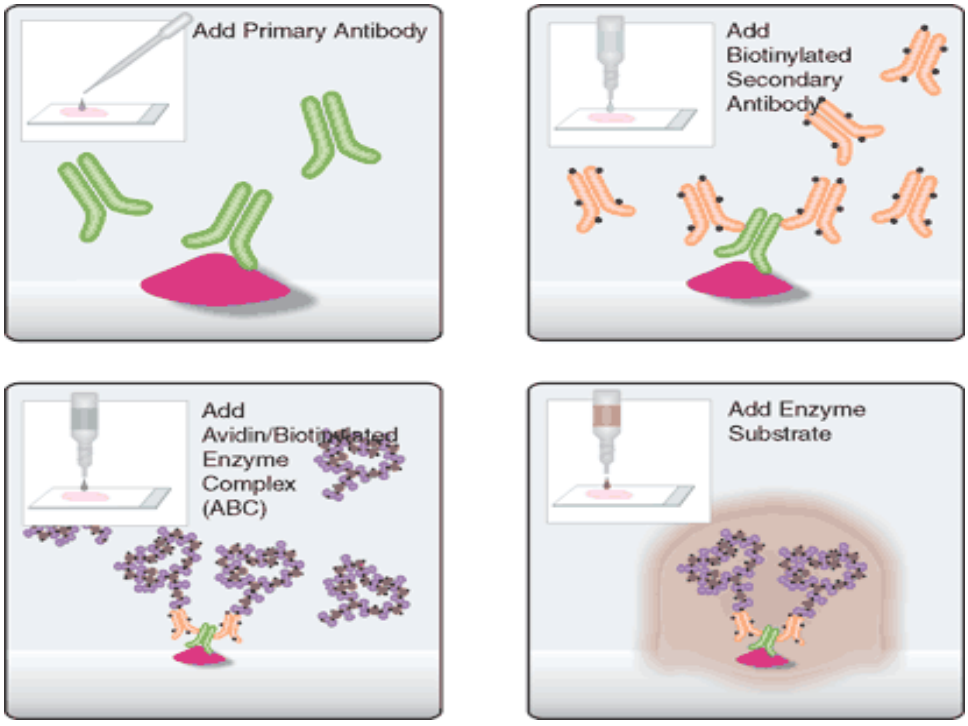


Figure 6 Procedure of ABC method for IHC. *The technique involves three layers. The first layer is unlabeled primary antibody. The second layer is biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by the DAB or other substrate to produce different*

colored end products

2.2.4.2 Immunohistochemical staining procedure

2.2.4.2.1 Deparaffinization and antigen retrieval

• All slides were labeled clearly with a pencil, noting antibody and dilution.

• Deparaffinization and rehydration were performed as follows: Two times for 10 minutes in xylene; two times for 5 minutes in 100% ethanol; one time for 2 minutes in 80% ethanol; one time for 1 minute in 80% ethanol; one time for 2 minutes in 70% ethanol; and once for 1 minute in 50% ethanol.

• To facilitate the immunological reaction of antibodies with antigens in fixed tissue, it is necessary to "retrieve" the antigens through pretreatment of the specimens. Antigen retrieval has been shown to increase reactivity of the majority of antigens in tissues. In the present study, sections were immersed in antigen retrieval solution (10 mM citrate acid, pH 6) and boiled for 15 minutes in a pressure cooker. The pressure cooker was brought to full pressure as quickly as possible and the heating times measured exactly from this point.

• At the end of the heating time, the pressure was released. As soon as possible the hot buffer was flushed out with cold water. The specimens were then washed and cooled for 10 minutes.

• The sections were rinsed in PBS for seconds.

• The sections were immersed in 0.2% Triton for 10 minutes. .

• And then, the sections were placed in modified endogenous oxidation blocking solution (3% hydrogen peroxide).

• At last, the slides were rinsed once for 5 minutes in PBS.

2.2.4.2.2 Blocking and staining

- The sections were incubated with one drop of Avidin reagent for 15 minutes.

- The slides were rinsed three times for 5 minutes each time in PBS.

- The sections were incubated with one drop of Biotin reagent for 15 minutes.

- The slides were rinsed three times for 5 minutes each time in PBS.

- The sections were incubated in 5% normal serum diluted in 5% BSA for one hour at room temperature to reduce non-specific binding of antibody. The incubation was performed in a sealed humidity chamber to prevent air-drying of the tissue sections. (The choice of blocking serum is the same as the species of the biotinylated secondary antibody).

- The slides were rinsed once for 5 minutes in PBS.

- The sections were covered with primary antibody diluted in 5% normal serum diluted in 5% BSA, and incubated at room temperature for 1 hour

- The sections were rinsed three times for 5 minutes in PBS, shaking gently.

- The sections were covered with diluted biotinylated secondary antibody in 5% BSA for 1 hour at room temperature in the humidity chamber.

- The sections were rinsed once for 5 minutes in PBS, shaking gently.

- The sections were incubated for 1 hour at room temperature in Vectastain ABC reagent (as described above).

- At last, the sections were rinsed once for 5 minutes in PBS.

2.2.4.2.3 Development and counterstaining

• The sections were incubated for approximately 1 to 7 minutes in DAB solution made up immediately prior to use.

• The slides were rinsed well for 10 minutes in running water.

• The sections were counterstained with Mayer's haematoxylin for 3 minutes.

• The slides were rinsed for 10 minutes in running water.

• The slides were differentiated in alcoholic-hydrochloric acid solution for 1 to 3 seconds.

• Then, the slides were rinsed for 10 minutes in running water.

• At last, the slides were covered with coverslips. .

2.2.4.2.4 Concentration of antibodies and DAB substrate incubation time

Initially, common slides of non small cell lung cancer tissues were tested to determine the antibody dilution and DAB substrate incubation time. The optimal concentrations for the specific antibodies were obtained and successfully used in our NSCLC TMAs. The antibody dilution and developing times are shown in Table 4.

Antibody	Dilution	DAB developing time (minutes)
• Anti-Rad51 (clone 1G8)	1:5 000	1
• Anti-Ape/Ref-1 (clone 13B8E5C2)	1:5 000	1
• Anti-p53 (Ab-6, clone DO-1)	1:500	7
• Anti-Ki-67 (Ab1, clone k-3)	1:200	7
• Anti-HER2 (code No. A 0485)	1:200	7
• Anti-E-cadherin (clone 4A2C7)	1:100	7
• Anti-ß-catenin (clone CAT-5H10)	1:100	7

Table 4 Antibody dilution and DAB developing time of involved antibodies

2.2.4.2.5 Control of IHC staining

In the present study, positive and negative controls were set up to control the quality of IHC. Known positive tissues for specific markers were used as a positive control.

As negative control, normal lung tissue and tissues of benign lung diseases present on the tissue microarray were used as internal negative control. Known positive tissues stained with normal serum (the same species as primary antibody) replacing the primary antibody was used as external negative control.

2.2.5 Assessment of IHC for TMAs

Stained tissue microarray slides were evaluated under a light microscope. Pictures were captured using a high-resolution (4k X 3k pixels) digital camera

The immunostaining of TMA slides were assessed by two pathologists without being aware of the clinical, pathological and follow up data. The nonmalignant and malignant tissues were scored for marker expression by assessing the site of positive staining. In case of disagreement the slides were reassessed.

Because of tumor heterogeneity, the tissue sections were scored semi-quantitatively under the light microscopy. The concept of PCI (positive stained cell index), which means the proportion of positively stained tumor cells, was adapted for the analyses of the results in this study. Additionally, for markers with membranous and cytoplasmic expression, intensity of positivity was considered in the assessment of the sections as well, because the intensity of positivity in membranous and cytoplasmic expression varied widely among samples. At least 200 tumor cells were counted to calculate the PCI in each specimen on TMA.

Because this study was stressed on the prognostic role of molecular tumor markers, an optimal cut-off point of marker index was determined that allowed best separation of patients into those with favourable and those with unfavourable clinical features and prognosis [Kronqvist et al., 1998]. For this procedure, we initially described the distribution of PCI and positive intensity of marker expression, and then selected some candidate for best cut-off point values around median or mean of PCI and positive intensity. Ultimately, the corresponding *p* values of prognostic significance for all candidates of cut-off points were calculated, and the cut-off point representing the highest statistical significance (i.e. the lowest *p* value) was regarded as the optimal threshold to separate prognostic different groups. Usually, we called cases whose IHC scores less than cut-off point as “low level expression”, whereas those more than cut-off point as “high level expression”.

The cut-off points of markers included in this study are introduced and listed in the results section.

2.2.6 Statistical analysis

All statistical analyses were performed using the SPSS 10.0 statistical software package (SPSS Inc., Chicago, IL). Correlations between antigen expression and given categorized parameters were evaluated using the nonparametric Mann-Whitney U test (for two categories) or Kruskal-Wallis test (for multiple categories). Kaplan-Meier curves were plotted from overall survival data, and the log-rank test was used for analysis of differences between patients with different level of markers expression.

Patients, who were still alive or lost to follow-up or died of other causes than lung cancer recurrence or metastasis before January 2003, were treated as censored data in the survival analyses. Multivariate survival analysis was performed using the Cox Proportional Regression Hazard Model. For multivariate analysis, all variables were

selected in a stepwise fashion (forward selection of covariates) to evaluate the predictive power of each variable independently of the others. A *P* value of 0.1 was adopted as the limit for entering and removing covariates. The relative risks (RR) and the associated 95% confidence intervals were calculated for the prognostic factors that contributed significantly to the model. For all analyses, statistical significance was assumed at a *P* level of <0.05.

All statistical analyses were performed under the guidance of a clinical epidemiologist and statistician, Ph. D. Wenzhao Zhong, at the Lung Cancer Research Institute of Guangdong province, P.R. China.

3. Results

3.1 Clinico-pathologic variables

Three hundred and eighty-three patients suffering from NSCLC were included in this study. Demographic, clinical and histopathological data, including age, sex, histology, TNM stage, surgical procedure, differentiation and primary tumor site, were collected retrospectively. Unfortunately, Data on smoking history were missing for the majority of patients, so that any analysis on the relationship between smoking and other clinicopathologic parameters and survival could not be done.

The median age of the patients was 59 years (range, 22 to 94 years) at the time of diagnosis (Figure 7), and the majority of the patients were male (71.3%). In the majority of cases lobectomy was performed (301 of 383 patients); 54 patients underwent pneumonectomy; 28 patients received conservative lung resection. Complete resection

was done for 343 patients, incomplete resection for 40 patients. Incomplete resection means either macroscopic evidence of the tumor or metastatic lymph nodes left behind, microscopic evidence of the tumor on the resected stump, or clinical evidence of a distant metastasis.

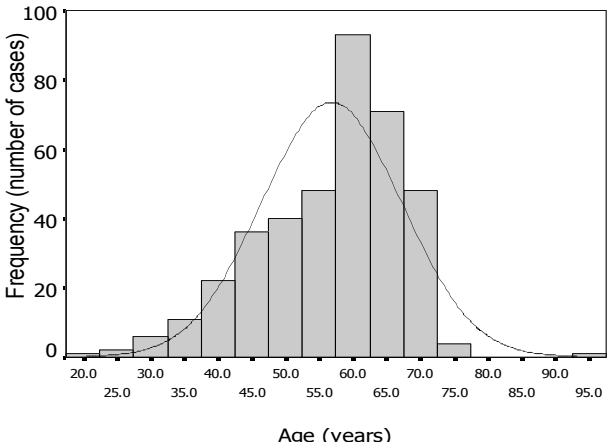


Figure 7. Distribution of age of 383 NSCLC patients

Histopathological examination of resected tumors revealed that adenocarcinoma was the dominant histological group (192 cases), 132 cases were squamous cell carcinomas, 44 adenosquamous cell carcinomas, 2 large cell carcinomas, 5 lung sarcomas and 8 were other less common malignant neoplasms including carcinoid tumor, adenoid cystic carcinoma and mucoepidermoid.

Postoperative staging evaluation demonstrated that 110 had stage I disease, 91 had stage II disease, 124 had stage IIIA disease, 37 had stage IIIB disease, and 21 had stage IV disease. Twenty-one had T_1 disease, 194 T_2 disease, 130 had T_3 disease, and 38 had T_4 disease. One hundred and seventy one had N_0 disease, 78 had N_1 disease, 126 had N_2 disease, and 8 had N_3 disease.

Two hundred and nine tumors were located in the upper lobe, 31 were in the middle lobe, 130 were in the lower lobe and 13 were mixed or hard to be distinguished.

3.2 Clinicopathologic variables and clinical outcome

Univariate analyses of the demographic and clinical variables revealed that tumor status (T status), lymph node status (N status), metastasis status (M status), clinical stage and tumor cell differentiation all have prognostic significance with regard to overall survival. The results are presented in Table 5.

variables	Patients (cases)	Mean survival (months)	Median survival (months)	5-Year Survival (%)	*p*-value
Age					
<59 years	184	55	45	44	0.974
⩾59 years	199	55	37	44	
Sex					
Male	273	55	39	45	0.865
Female	110	55	34	42	
T status					
1	21	84	n.r.[1]	75	<0.001
2	194	61	49	47	
3	130	50	31	41	
4	38	29	17	21	
N status					
0	171	72	n.r.[1]	62	<0.001
1	78	57	45	48	
2	126	29	22	16	
3	8	21	16	0	
M status					
0	362	56	42	45	0.041
1	21	37	25	20	

Stage					
Ⅰ	110	79	n.r.[1]	67	<0.001
Ⅱ	91	59	62	54	
Ⅲ	161	36	22	23	
Ⅳ	21	37	25	19	
Histology					
Squamous carcinoma	132	60	59	49	0.448
Adenocarcinoma	192	52	34	41	
adenosquamous	44	47	33	41	
Others[2]	15	57	46	47	
Differentiation					
Poor	70	36	15	28	<0.001
Moderate	183	54	37	40	
Well	130	68	78	59	
Surgical procedure					
Lobectomy	301	57	45	46	0.222
Pneumonectomy	54	45	31	40	
Conservative resection[3]	28	37	32	26	

Table 5. Predictive variables for prognosis of 383 NSCLC patients by univariate analysis (log-rank test)

Abbreviations: *[1] n.r.=not reached. [2] others include adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.[3] conservative resections include segmentectomy and wedge resection*

3.3 Expression of Rad51, Ape/Ref-1, p53, Ki67, HER2, E-cadherin and catenin in NSCLC.

3.3.1 Scoring of Immunohistochemistry

The TMA sections were scored semiquantitatively for immunohistochemical staining by light microscopy. For the markers presenting nuclear expression, Rad51, Ape/Ref-1, p53 and Ki67, expression level was classified into two groups according to cut-off points, namely high level expression and low level expression. On the single cell level, tumor cells with clear nuclear staining were scored as positive, tumor cells with weak or no nuclear staining as negative. The percentage of tumor cells in a given tumor classified as positively stained is then used to evaluate the respective antigen for its use a molecular marker in NSCLC. The cut-off point, which can best separate cases into different groups according to survival outcome, is a PCI value. In this study, cut-off

points for Rad51, Ape/Ref-1, p53 and Ki67 were determined as PCI=10%, PCI=50%, PCI=10%, PCI=10%, respectively (Table 6).

Markers	Low level expression	High level expression
Rad51	PCI<10% (stained tumor cells)	PCI≥10% (stained tumor cells)
Ape/Ref-1	PCI≤50% (stained tumor cells)	PCI>50% (stained tumor cells)
p53	PCI<10% (stained tumor cells)	PCI≥10% (stained tumor cells)
Ki67	PCI≤10% (stained tumor cells)	PCI>10% (stained tumor cells)

Table 6. Expression levels of the markers with nuclear positive staining

On the other hand, cut-off point values were determined combining PCI plus intensity of positivity for markers presenting membranous or cytoplasmic expression, HER2, E-cadherin and ß-catenin, because the intensity of positivety in membranous and cytoplasmic expression varied widely among samples. The detailed information about the classification system for these markers will be given in the corresponding chapters.

3.3.2 Rad51 expression in NSCLC

3.3.2.1 Pattern of Rad51 expression in NSCLC

Alteration of Rad51 expression has been reported in several human malignancies [Maacke et al 2000]. In order to evaluate pattern of Rad51 expression in NSCLC, immunohistochemistry was performed on TMAs. Immunochemical staining revealed 235 informative cases which could be assessed and scored under the light microscope. Positive tumor cells generally showed a nuclear staining pattern in NSCLC.

The expression status of Rad51 was classified into 2 groups: (1) low level expression: PCI <10%; (2) high level expression: PCI≥10%.

Figure 8 shows the frequency distribution of Rad51 PCI in 235 NSCLC specimens. Figure 9 illustrates representative examples of the different expression level of Rad51 staining in squamous cell carcinoma and adenocarcinoma of NSCLC. Figure 10 gives

the high order magnification pictures of figure 9b and 9d

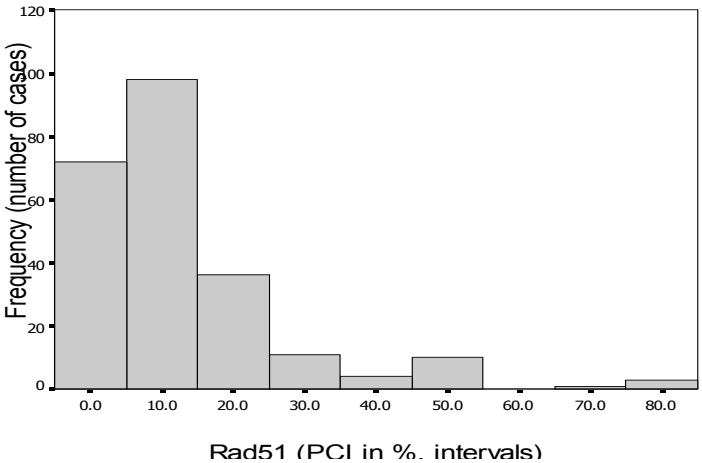
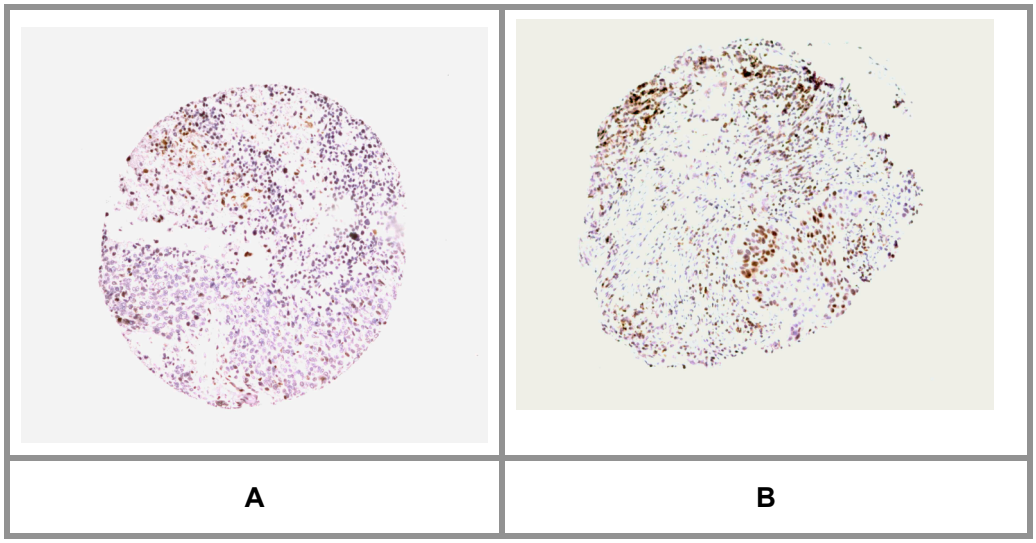


Figure 8. Frequency distribution of Rad51 PCI



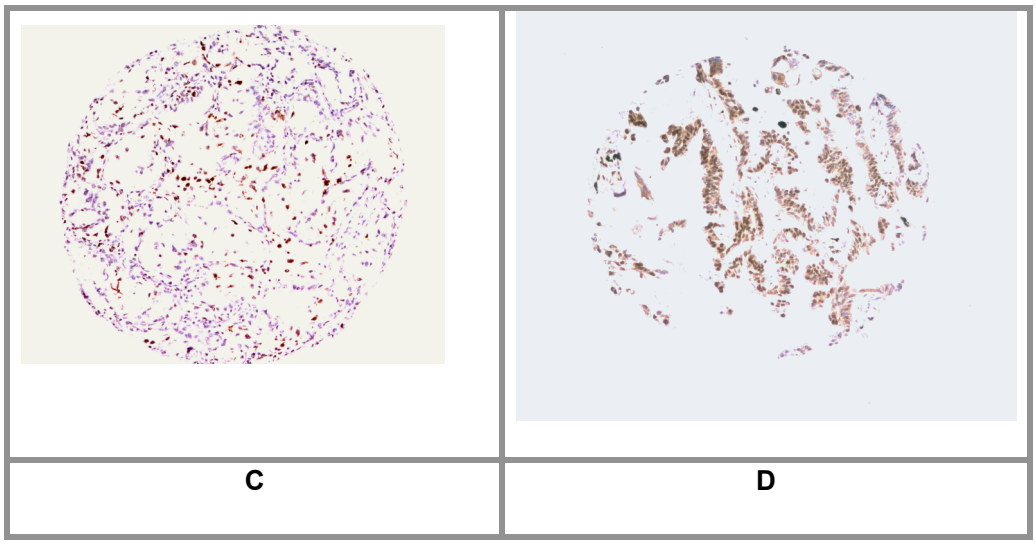


Figure 9. Expression of Rad51 by immunohistochemical analysis, magnification 1×10**.** *A, low level expression of Rad51 in squamous cell carcinoma (PCI<10%); B, high level expression of squamous cell carcinoma (PCI=60%); C, low level expression of adenocarcinoma (PCI<10%); D, high level expression of adenocarcinoma (PCI=80%)*

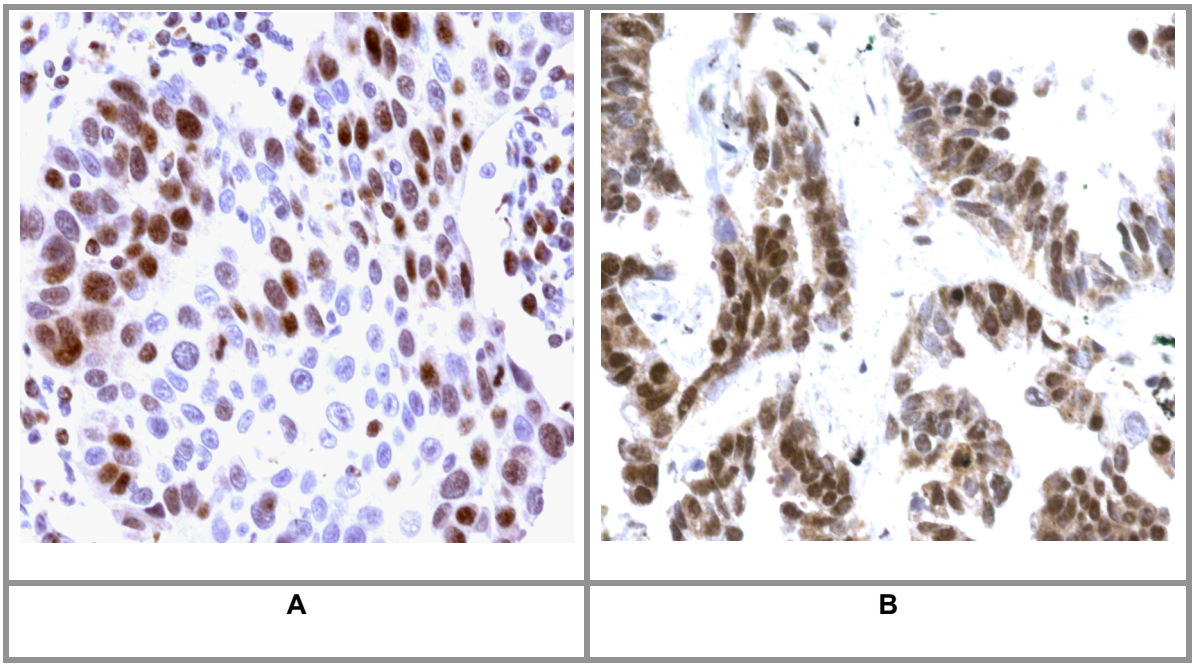


Figure 10. High order magnification of figure 9b *(A)* **and 9d** *(B), magnification 10×40.*

3.3.2.2 Correlation between Rad51 expression and clinicopathological parameters in NSCLC

Rad51 expression status was correlated with cliniclopathological features. The

relationship between Rad51 expression and cliniclopathological features in NSCLC is shown in Table 7. Sixty-five of 235 (27.7%) NSCLC cases showed high-level expression of Rad51; including 19 of 74 (25.7%) squamous cell carcinoma (SCC) and 38 of 123 (30.5%) adenocarcinoma (ADC).

We found no significant correlation between Rad51 expression and age, sex, tumor status (T status), lymph node status (N status), metastasis status (M status), clinical stage, tumor cell type and differentiation in NSCLC using the nonparametric Mann-Whitney U test (for two categories) or Kruskal-Wallis test (for multiple categories).

Parameters	**Rad51 (cases and percentage)**		*p*-value
	-[1]	+[2]	
Total	170 (72.3%)	65 (27.7%)	
Age			
<59 years	82 (76.6%)	25 (23.4%)	0.178
⩾59 years	88(68.8%)	40 (31.2%)	
Sex			
Male	127 (73.4%)	46(26.6%)	0.540
Female	43(69.4%)	19(30.6%)	
T status			
1	9(60.0%)	6 (30.0%)	0.595
2	89(74.8%)	30 (25.2%)	
3	56(72.7%)	21 (27.3%)	
4	16 (66.7%)	8 (33.3%)	
N status			
0	75(70.8%)	31(29.2%)	0.143
1	40(83.3%)	8(16.7%)	
2	52(66.7%)	26(33.3%)	
3	3(100%)	0	
M status			
0	164(73.5%)	59(26.5%)	0.076

1	6(50.0%)	6(50.0%)	
Stage			
Ⅰ	50(75.8%)	16(24.2%)	0.334
Ⅱ	41(73.2%)	15(26.8%)	
Ⅲ	73(72.3%)	28(27.7%)	
Ⅳ	6(50.0%)	6(50.0%)	
Histology			
SCC[3]	55(74.3%)	19(25.7%)	0.394
ADC[4]	85(69.1%)	38(30.9%)	
ADSCC[5]	25(83.3%)	5(16.7%)	
Others[6]	5(62.5%)	3(37.5%)	
Differentiation			
Poor	36(69.2%)	16(30.8%)	0.535
Moderate	74(70.5%)	31(29.5%)	
Well	60(76.9%)	18(23.1%)	

Table 7. Relation between Rad51 expression and clinicopathological parameters in 235 cases of NSCL

Abbreviations:*[1] – low level expression of Rad51; [2] +high level expression of Rad51; [3]SCC, squamous cell carcinoma; [4]ADC, adenocarcinoma; [5]ADSCC, adenosquamous carcinoma; [6]others include adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.*

3.3.3 Ape/Ref-1 expression in NSCLC

3.3.3.1 Pattern of Ape/Ref-1 expression in NSCLC

High-level expression of the multifunctional protein Ape/Ref-1 has been observed in many human tissues [Puglisi et al., 2002]. To investigate the pattern of Ape/Ref-1 in NSCLC, we immunohistochemically stained NSCLC TMAs with antibody against Ape/Ref-1. The results revealed 348 informative cases which could be evaluated.

The predominant pattern of Ape/Ref-1 expression observed in this series of NSCLC was nuclear, although some of tumor cells showed either cytoplasmic or both nuclear and cytoplasm staining. The nuclear expression status of Ape/Ref-1 was assessed according to the following criteria: (1) low level expression: PCI $\leq$50%; (2) high level expression: PCI>50%.

Figure 11 shows the frequency distribution of Ape/Ref-1 PCI in 348 NSCLC specimens. Figure 12 demonstrates the different expression level of Ape/Ref-1 in squamous cell carcinoma and adenocarcinoma of NSCLC. Figure 13 shows high order magnification pictures of Figures 12b and 12d

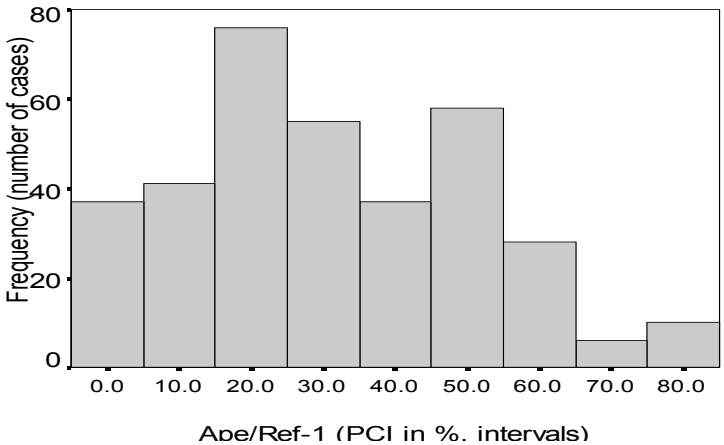
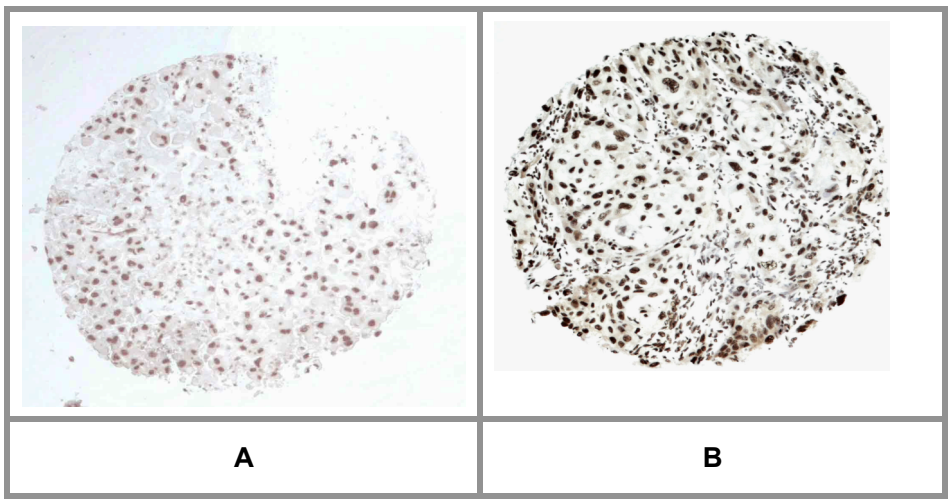


Figure 11. Frequency distribution of Ape/Ref-1 PCI



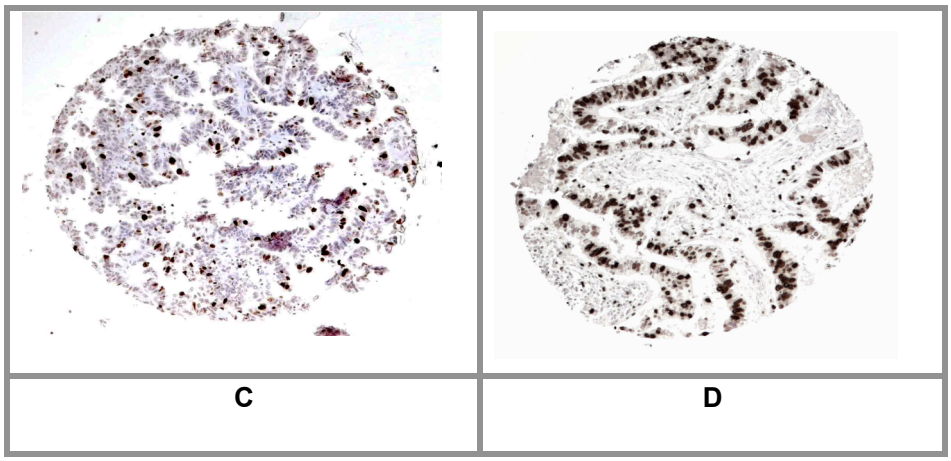


Figure 12. Expression of Ape/Ref-1 by immunohistochemical analysis, magnification 1×10**.** *A, low level expression of Ape/Ref-1 in squamous cell carcinoma (PCI=20%); B, high level expression of Ape/Ref-1 in squamous cell carcinoma (PCI=80%); C, low level expression of Ape/Ref-1 in adenocarcinoma (PCI=20%); D, high level expression of Ape/Ref-1 in adenocarcinoma (PCI=80%)*

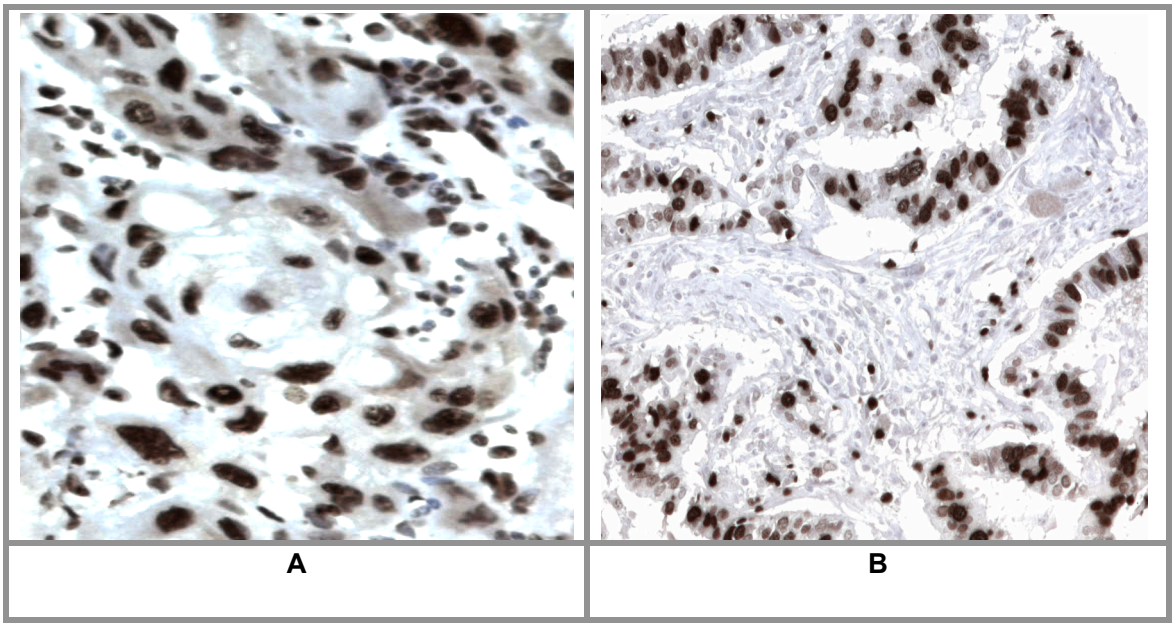


Figure 13. High order magnification of figure 12b *(A)* **and 12d** *(B), magnification 10×40.*

3.3.3.2 Correlation between Ape/Ref-1 expression and clinicopathological parameters in NSCLC

Numerous investigators have found that Ape/Ref-1 was related to tumour progression [Puglisi et al., 2002]. However, little is known about the relationship between expression

of Ape/Ref-1 protein and clinicopathological features in NSCLC.

The expression frequency of Ape/Ref-1 protein in NSCLC is shown in Table 8. One hundred of 348 (28.7%) NSCLC showed low level expression of Ape/Ref-1; including 27 of 120 (59.1%) SCC and 59 of 179 (30.3%) ADC. No statistical correlation was found between Ape/Ref-1 expression and age, sex, tumor status (T status), lymph node status (N status), metastasis status (M status), clinical stage and tumor cell type. However, the frequency of low level nuclear expression of Ape/Ref-1 was significantly higher in the group of well differentiated tumors compared to the moderately and poorly differentiated tumor groups (p=0.028).

Parameters	**Ape/Ref-1 (cases and percentage)**		*p*-value
	-[1]	+[2]	
Total	100(28.7%)	248 (71.3%)	
Age			
<59 years	43 (26.1%)	122(73.9%)	0.295
≥59 years	57 (31.1%)	126(68.9%)	
Sex			
Male	67(26.8%)	183 (73.2%)	0.942
Female	33(33.7%)	65(66.3%)	
T status			
1	10 (47.6%)	11(52.4%)	0.259
2	43 (24.6%)	132(75.4%)	
3	29 (25.2%)	86(74.8%)	
4	18 (48.6%)	19 (51.4%)	
N status			
0	44(27.3%)	117(72.7%)	0.671
1	20(31.7%)	43(68.3%)	
2	37(31.6%)	80(68.4%)	
3	1(14.3%)	6(85.7%)	
M status			
0	96(29.0%)	235(71.0%)	0.627
1	4(23.5%)	13(76.5%)	

Stage			
Ⅰ	27(25.7%)	78(74.3%)	0.869
Ⅱ	17(22.1%)	60(77.9%)	
Ⅲ	52(34.9%)	97(65.1%)	
Ⅳ	4(23.5%)	13(76.5%)	
Histology			
SCC[3]	27(22.5%)	93(77.5%)	0.275
ADC[4]	59(33.0%)	120(67.0%)	
ADSCC[5]	10(27.8%)	26(72.2%)	
Others[6]	4(30.8%)	9(69.2%)	
Differentiation			
Poor	26(22.2%)	91(77.8%)	0.028
Moderate	46(28.4%)	116(71.6%)	
Well	28(40.6%)	41(59.4%)	

Table 8. Relation between Ape/Ref-1 expression and clinicopathological parameters in 348 cases of NSCLC

Abbreviations: *[1]-low level expression of Ape/Ref-1; [2] +high level expression of Ape/Ref-1; [3]SCC, squamous cell carcinoma; [4]ADC, adenocarcinoma; [5]ADSCC, adenosquamous carcinoma; [6]others include adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.*

3.3.4 p53 expression in NSCLC

3.3.4.1 Pattern of p53 expression in NSCLC

Accumulation of p53 protein is a characteristic feature of various human malignancies. In this study, immunohistochemical staining revealed 360 informative cases which could be distinguished and scored under a light microscope. Immunoreactivity for p53 was found only in the nuclear compartment of neoplastic cells in NSCLC.

The expression status of p53 was assessed according to PCI. Scoring criteria for p53 were as follows: (1) low level expression: PCI<10%; (2) high level expression: PCI≥10%.

Figure 14 shows the frequency distribution of p53 PCI in 360 NSCLC specimens. Figure 15 demonstrate the different level of p53 staining in squamous cell carcinoma and

adenocarcinoma of NSCLC. Figure 16 provides the high order magnification pictures of Figures 15b and 15d.

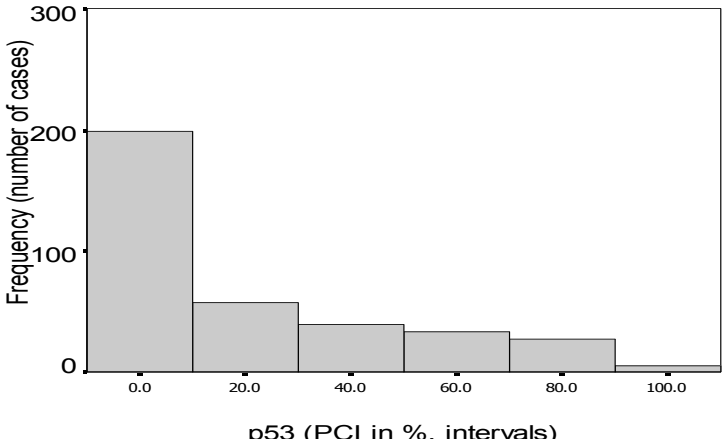
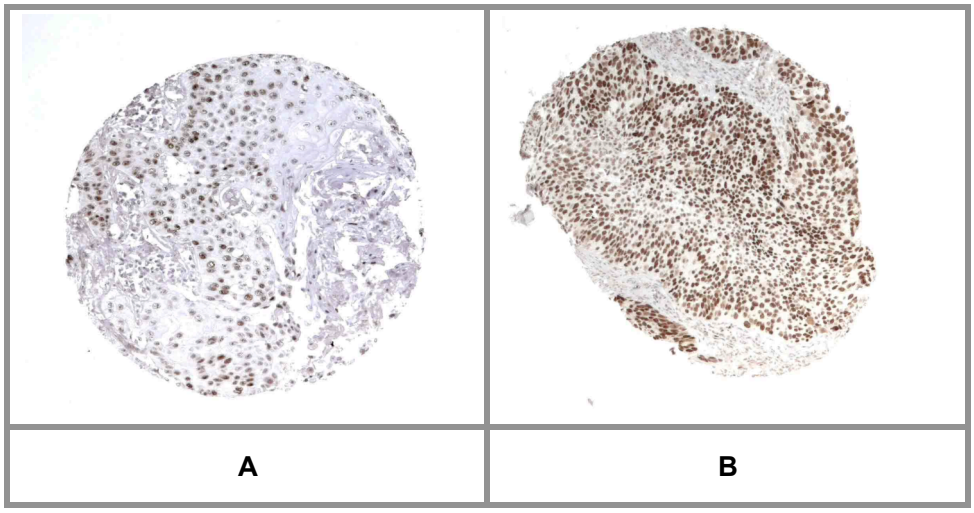


Figure 14. Frequency distribution of p53 PCI



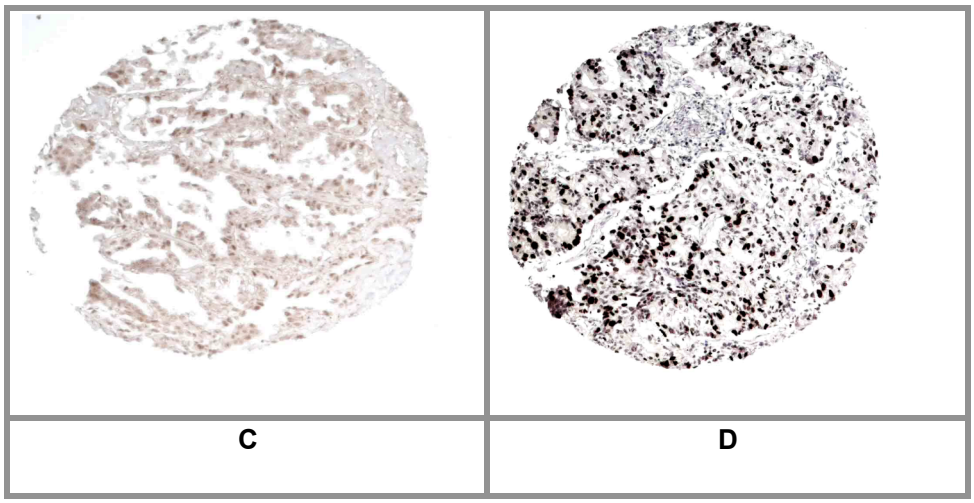


Figure 15. Expression of p53 by immunohistochemical analysis, magnification 1×10. *A ,low level expression of p53 in squamous cell carcinoma (PCI=10%); B, high level expression of p53 in squamous cell carcinoma (PCI=90%); C, low level expression of p53 in adenocarcinoma (PCI=10%); D, high level expression of p53 in adenocarcinoma (PCI=70%).*

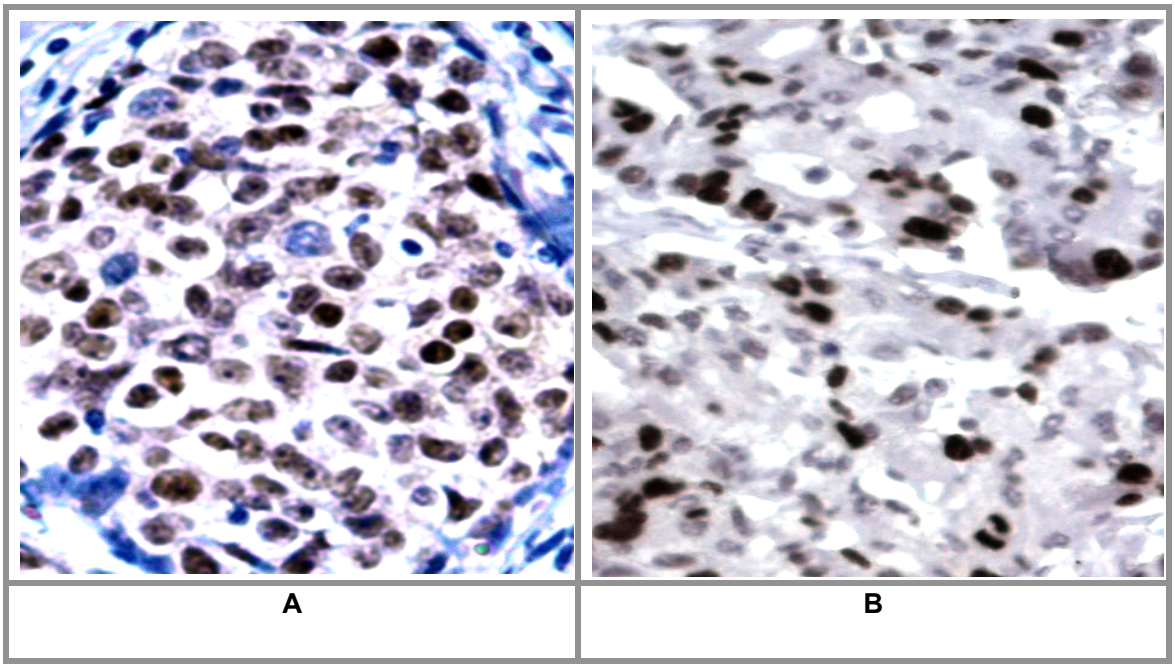


Figure 16. High order magnification of figure 15b *(A)* **and 15d** *(B), magnification 10×40.*

3.3.4.2 Correlation between p53 expression and clinicopathological parameters in NSCLC

In order to evaluate the relationship between status of the p53 protein and

clinicopathological features in NSCLC, p53 expression was correlated with clinicopathological parameters.

The expression frequency of p53 protein in NSCLC is shown in Table 9. One hundred and thirty three of 360 (36.9%) NSCLC showed high level expression of p53; including 58 of 128 (45.3%) SCC and 57 of 179 (31.8%) ADC. No statistical correlation was found between p53 expression and sex, T status, N status, M status, clinical stage and tumor cell type. In contrast, the frequency of p53 expression was significantly higher in poorly differentiated tumors compared to moderately and well-differentiated tumors (p=0.009). A significant association was also found between p53 expression level and the age of the patients (p=0.030).

Parameters	**p53 (cases and percentage)**		*p*-value
	-[1]	+[2]	
Total	227 (63.1%)	133 (36.9%)	
Age			
<59 years	119 (68.8%)	54 (31.2%)	0.030
≥59 years	108 (57.8%)	79 (42.2%)	
Sex			
Male	156 (60.2%)	103(39.8%)	0.075
Female	71(70.3%)	30(29.7%)	
T status			
1	14(66.7%)	7 (33.3%)	0.271
2	119(65.4%)	63 (34.6%)	
3	67(56.3%)	52 (43.7%)	
4	27 (71.1%)	11 (28.9%)	
N status			
0	102(64.2%)	57(42.8%)	0.111
1	49(68.1%)	23(31.9%)	
2	74(61.2%)	47(38.8%)	
3	2(25.0%)	6(75.0%)	
M status			
0	215(63.4%)	124(36.6%)	0.563
1	12(57.1%)	9(42.9%)	

Stage			
Ⅰ	70(66.7%)	35(33.3%)	0.714
Ⅱ	51(64.6%)	28(35.4%)	
Ⅲ	94(60.6%)	61(39.4%)	
Ⅳ	12(57.1%)	9(42.9%)	
Histology			
SCC[3]	70(54.7%)	58(45.3%)	0.106
ADC[4]	122(68.2%)	57(31.8%)	
ADSCC[5]	26(65.0%)	14(35.0%)	
Others[6]	9(69.2%)	4(30.8%)	
Differentiation			
Poor	31(47.0%)	35(53.0%)	0.009
Moderate	117(68.4%)	54(31.6%)	
Well	79(64.2%)	44(35.8%)	

Table 9. Relation between p53 expression and clinicopathological parameters in 360 cases of NSCLC

Abbreviations: *[1] – low level expression of p53; [2] + high level expression of p53; [3]SCC, squamous cell carcinoma; [4]ADC, adenocarcinoma; [5]ADSCC, adenosquamous carcinoma; [6]others include adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.*

3.3.5 Ki67 expression in NSCLC

3.3.5.1 Pattern of Ki67 expression in NSCLC

Expression of Ki67 has been used to assess tumor cell proliferation for example in breast cancers, lung tumors, nervous system tumors, and non-Hodgkin's lymphomas, etc. In the present study, 341 informative cases of NSCLC were immunohistochemically evaluated under a light microscope. Similar to p53 protein accumulation, immunoreactivity for Ki67 was found only in the nucleus of neoplastic cells.

The expression status of Ki67 was assessed according to PCI. Scoring criteria for Ki67 were as follows: (1) low level expression: PCI≤10%; (2) high level expression: PCI ＞10%.

Figure 17 shows the frequency distribution of Ki67 PCI in 341 NSCLC specimens.

Figure 18 demonstrates the different expression levels of Ki67 staining in squamous cell carcinoma and adenocarcinoma of NSCLC. Figure 19 shows high order magnification pictures of Figures 18b and 18d.

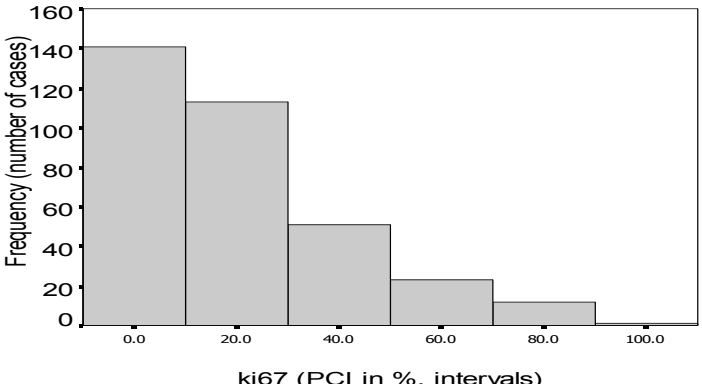
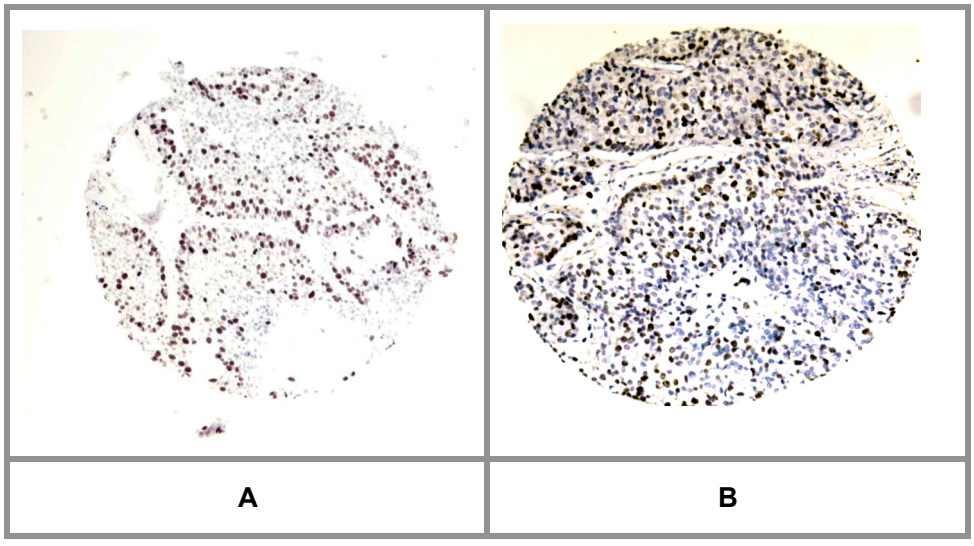


Figure 17 Frequency distribution of Ki67 PCI



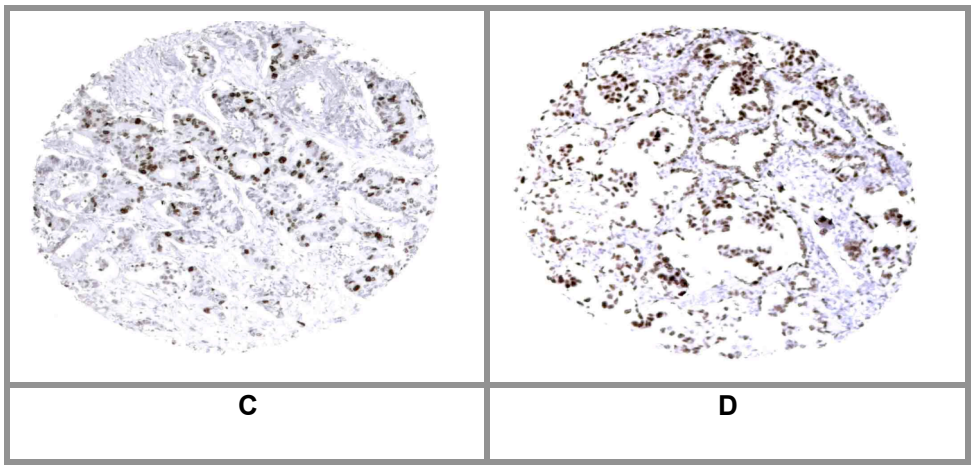


Figure 18. Expression of Ki67 by immunohistochemical analysis, magnification 1×10. *A, low level expression of Ki67 in squamous cell carcinoma (PCI=10%); B, high expression of Ki67 in squamous cell carcinoma (PCI=900%); C, low expression of Ki67 in adenocarcinoma (PCI=10%); D, high expression of Ki67 in adenocarcinoma (PCI=70%)*

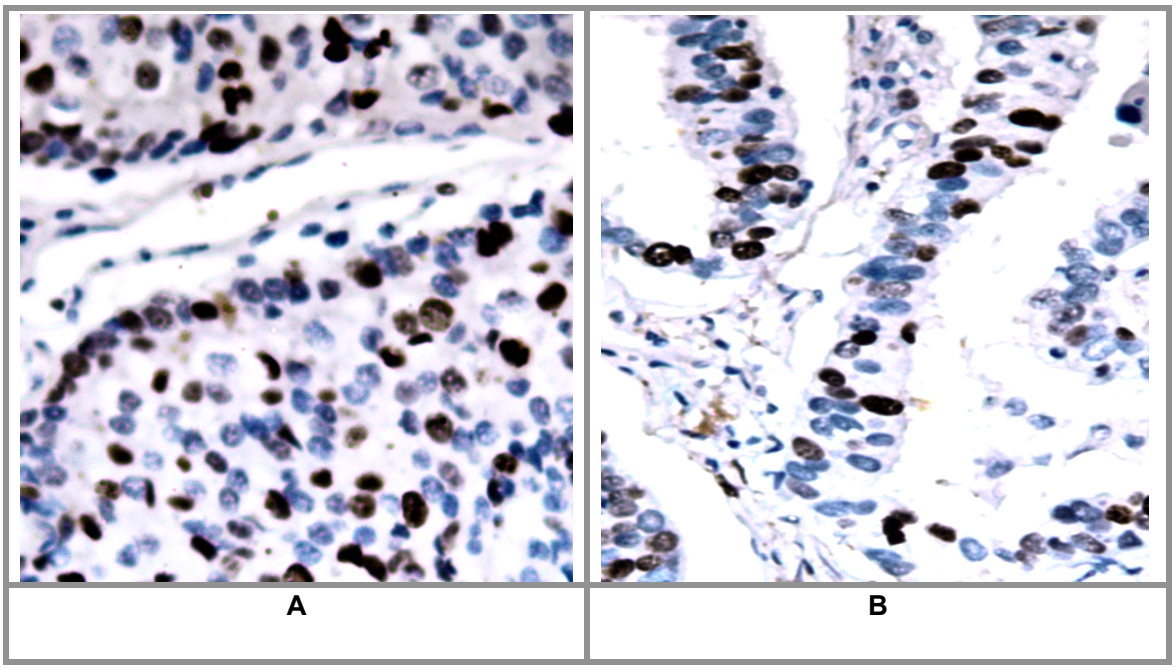


Figure 19. High order magnification of figure 18b *(A)* **and 18d** *(B), magnification 10×40.*

3.3.5.2 Correlation between Ki67 expression and clinicopathological parameters in NSCLC

Altered regulation of the cell cycle, which is related to tumor proliferative activity, is a hallmark of human cancers. The expression of Ki-67 antigen can be used as a simple histological marker for cell proliferation [Sherr, 1996]. For many tumor entities it is

known that the proliferation of tumor cells is related to stage and differentiation of the respective tumor. To evaluate the relationship between Ki67 expression level and clinicopathological features in NSCLC, Ki67 expression were correlated with clinicopathological parameters.

The expression frequency of Ki67 protein in NSCLC is shown in Table 10. One hundred and forty-four of 341 (42.2%) NSCLC exhibited high level expression of Ki67; including 68 of 115 (59.1%) SCC and 53 of 175 (30.3%) ADC.

No statistical correlation was found between Ki67 expression and age, tumor status (T status), lymph node status (N status), metastasis status (M status) and tumor cell differentiation, However, the frequency of Ki67 expression was significantly higher in squamous cell carcinoma and adenosquamous carcinoma of the lung than that in adenocarcinoma ($p<0.001$). Significant association was also found between Ki67 expression level and clinical stage, low level expression of Ki67 was more frequently in stage□than that in other stages ($p=0.009$).

Parameters	**Ki67 (cases and percentage)**		*p*-value
	-[1]	+[2]	
Total	197 (57.8%)	144 (42.2%)	
Age			
<59 years	93 (56.7%)	71 (43.3%)	0.702
⩾59 years	104(58.8%)	73 (41.2%)	
Sex			
Male	127 (52.3%)	116(47.7%)	0.001
Female	70(71.4%)	28(28.6%)	
T status			
1	14(66.7%)	7 (33.3%)	0.612
2	104(59.44%)	71 (40.6%)	
3	58(53.2%)	51 (46.8%)	
4	21 (58.31%)	15 (41.7%)	
N status			
0	96(62.7%)	57(37.3%)	0.117
1	38(52.8%)	34(47.2%)	
2	61(56.5%)	47(43.5%)	

3	2(25.0%)	6(75.0%)	
M status			
0	183(56.7%)	140(43.3%)	0.077
1	14(77.8%)	4(22.2%)	
Stage			
Ⅰ	68(68.7%)	31(31.3%)	0.009
Ⅱ	43(52.4%)	39(47.6%)	
Ⅲ	72(50.7%)	70(49.34%)	
Ⅳ	14(77.8%)	4(22.2%)	
Histology			
SCC[3]	47(40.9%)	68(59.1%)	0.000
ADC[4]	122(69.7%)	53(30.3%)	
ADSCC[5]	21(51.2%)	20(48.8%)	
Others[6]	7(70.0%)	3(30.0%)	
Differentiation			
Poor	40(63.5%)	23(36.5%)	0.420
Moderate	96(58.5%)	68(41.5%)	
Well	61(53.5%)	53(46.5%)	

Table 10. Relation between Ki67 expression and clinicopathological parameters in 341 cases of NSCLC

Abbreviations:*[1] – low level expression of Ki67; [2] + high level expression of Ki67; [3]SCC, squamous cell carcinoma; [4]ADC, adenocarcinoma; [5]ADSCC, adenosquamous carcinoma; [6]others include adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.*

3.3.6 HER2 expression in NSCLC

3.3.6.1 Pattern of HER2 expression in NSCLC

Immunohistochemical staining revealed 347 informative cases of NSCLC which could be evaluated under the light microscope. Tumor cells positive for HER2 generally showed a membrane and cytoplasmic staining pattern.

The expression status of HER2 was assessed combining PCI and staining intensity according to the criteria routinely used for clinical evaluation of HER2 expression at most hospitals and research centers worldwide [Amashita et al., 2004]. These criteria

divide HER2 expression into 4 categories: (1) 0: no staining is observed or staining is less than 10% of the tumor cells; (2) 1+: faint or barely perceptible staining is seen in more than 10% of the tumor cells; (3) 2+:, weak to moderate staining is observed in more than 10% of the tumor cells; (4) 3+: strong staining is observed in more than 10% of the tumor cells. Tumors with a score of 2+ or 3+ for HER2 were considered to be high-level expressors for HER2, and 0 or 1+ were considered to be low level expressors in the following relationship analyses.

Figure 20 shows the frequency distribution of HER2 staining levels in 347 NSCLC specimens. Figure 21 demonstrates the different levels of HER2 staining in squamous cell carcinoma and adenocarcinoma of NSCLC. Figure 22 provides high order magnification pictures of Figures 21d and 21h

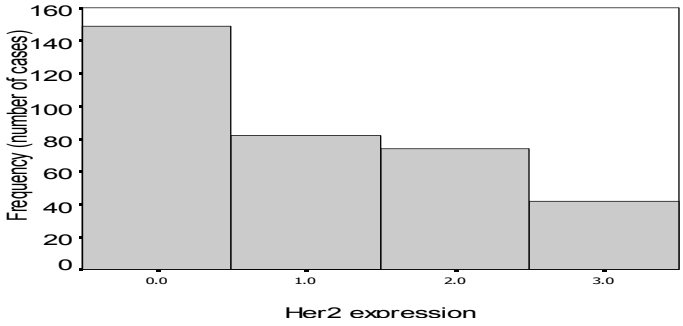
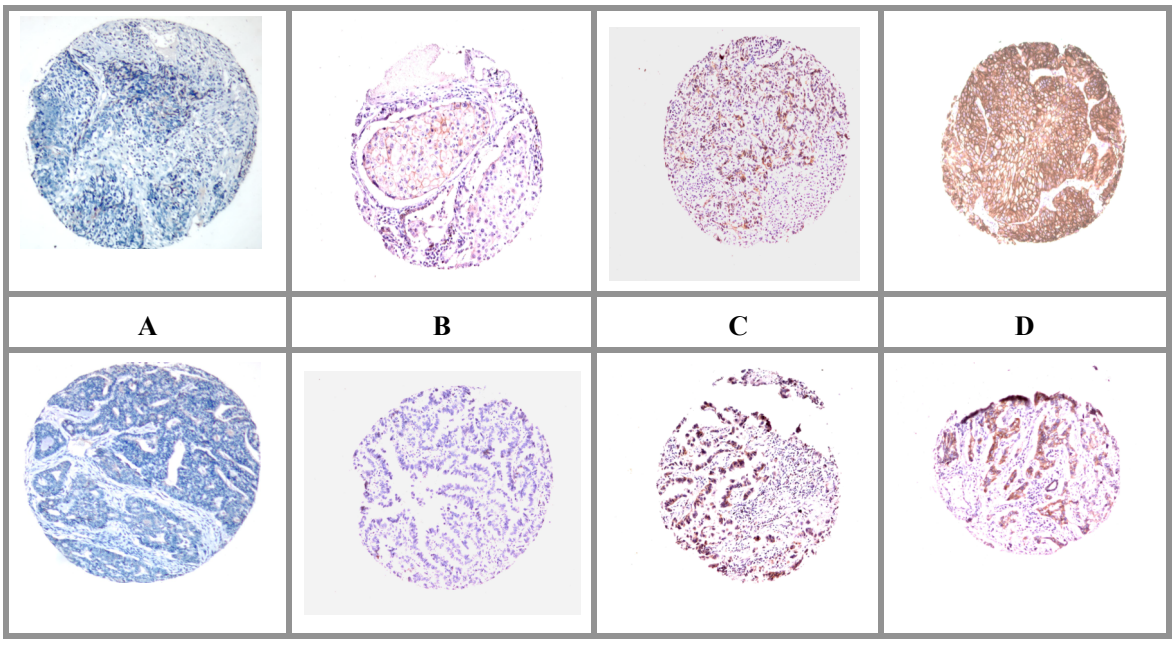
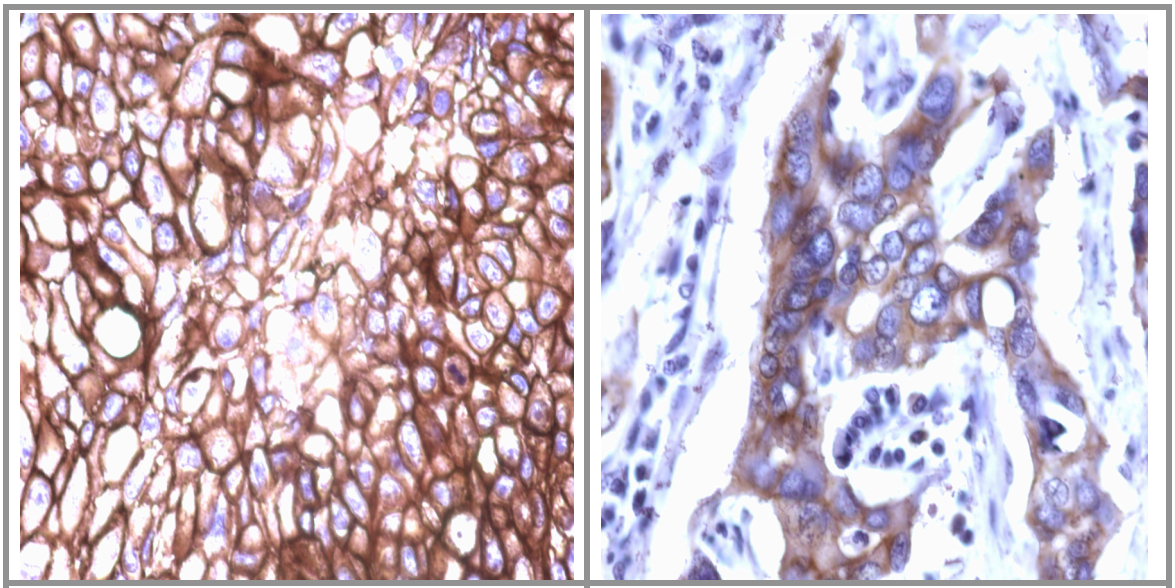


Figure 20. Frequency distribution of HER2staining



E	F	G	H

Figure 21. Expression of HER2 by immunohistochemical analysis, magnification 10×40. *A,0, no staining of HER2 in squamous cell carcinoma; B,1+, faint or barely staining of HER2 in squamous cell carcinoma; C, 2+,weak staining of HER2 in squamous cell carcinoma; D,3+, strong expression of HER2 in squamous cell carcinoma. E,0, no staining of HER2 in adenocarcinoma; F,1+, faint or barely staining of HER2 in adenocarcinoma ; G,2+, weak staining of HER2 in adenocarcinoma; H, 3+,strong staining of HER2 in adenocarcinoma*.



A B

Figure 22. High order magnification of Figure 21d (A) and Figure 21h (B), *magnification 10×40.*

3.3.6.2 Correlation between HER2 expression and clinicopathological parameters in NSCLC

The HER2 oncogene, which encodes the tyrosine kinase receptor and plays numerous roles in mammalian growth and development, is commonly over-expressed in several types of cancer. Moreover, Over-expression was detected in adenocarcinoma more frequently than in squamous cell carcinoma of NSCLC [Nakamura et al., 2003].

The expression frequency of HER2 protein in NSCLC is shown in Table 11. One

hundred and sixty of 347 (33.4%) cases of NSCLC showed high level expression of HER2; including 31 of 120 (25.8%) SCC and 68 of 178 (38.2%) ADC.

No statistical correlation was found between HER2 expression and age, sex, tumor status (T status), lymph node status (N status), metastasis status (M status), clinical stage and tumor cell type. However, the results showed that expression of HER2 was significantly related to the differentiation state of tumor cells. The frequency of HER2 expression was significantly higher in poorly differentiated tumor cells compared to moderately and well differentiated tumor cells ($p=0.035$).

Parameters	HER2 (cases and percentage)		*p*-value
	- or 1+[1]	2+ or 3+[2]	
Total	231 (66.6%)	116(33.4%)	
Age			
<59 years	115(67.3%)	56(32.7%)	0.791
⩾59 years	116(65.9%)	60 (34.1%)	
Sex			
Male	161 (65.4%)	85(34.6%)	0.489
Female	70(69.3%)	31(30.7%)	
T status			
1	10(50.0%)	10 (50.0%)	0.385
2	118(66.3%)	60 (33.7%)	
3	79(69.9%)	34 (30.1%)	
4	24 (66.7%)	12 (33.3%)	
N status			
0	108(68.8%)	49(31.2%)	0.755
1	47(68.1%)	22(31.9%)	
2	71(62.8%)	42(37.2%)	
3	5(62.5%)	3(37.5%)	
M status			
0	217(66.0%)	112(34.0%)	0.301
1	14(77.8%)	4(22.2%)	
Stage			
Ⅰ	67(65.0%)	36(35.0%)	0.869
Ⅱ	57(70.4%)	24(29.6%)	
Ⅲ	93(64.1%)	52(35.9%)	
Ⅳ	14(77.8%)	4(22.2%)	
Histology			
SCC[3]	89(74.2%)	31(25.8%)	0.063
ADC[4]	110(61.8%)	68(38.2%)	
ADSCC[5]	22(59.5%)	15(40.5%)	
Others[6]	10(83.3%)	2(16.7%)	
Differentiation			
Poor	35(53.8%)	30(46.2%)	0.035
Moderate	117(71.8%)	46(28.2%)	
Well	79(66.4%)	40(33.6%)	

Table 11. Relation between HER2 expression and clinicopathological parameters in 347 cases of NSCLC

Abbreviations:[1]*- or 1+: low level expression of HER2,* [2] *2+ or 3+: high level expression of HER2* [3]*SCC,*

squamous cell carcinoma; [4]ADC, adenocarcinoma; [5]ADSCC, adenosquamous carcinoma; [6]others include adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.

3.3.7 E-cadherin and ß-catenin expression in NSCLC

3.3.7.1 Pattern of E-cadherin and ß-catenin expression in NSCLC

E-cadherin and ß-catenin complex plays a major role in maintaining the intercellular junctions in epithelial tissues. Many investigations find that aberrant expression of E-cadherin and ß-catenin are associated with differentiation of tumor cells, clinical stage and metastasis in NSCLC [Bremnes et al., 2002a].

Immunochemical staining revealed 351 informative cases, which could be distinguished and scored under a light microscope. In the vast majority of normal epithelial cells and NSCLC tumor cells, the expression of E-cadherin and ß-catenin were localized mainly to membranes of the tumor cells, but in some cases it was localized in the cytoplasm as well.

Scoring criteria for E-cadherin expression are as follows: (1) low level expression: immunohistochemical staining exhibit in less than 50% of the tumor cell population; or even more than 50% of tumor cells are stained but the intensity of staining is faint; (2) intermediate expression: more than 50% but less than 80% of the tumor cells are stained; (3) high level expression: more than 80% of the tumor cells are stained. ß-catenin expression is classified into two groups: (1) low level expression: staining present in less than 50% of the tumor cell population; or even more than 50% of tumor cells stained but with faint staining intensity; (2) high level expression: more than 50% of the tumor cells are stained.

Figure 23 and Figure 24 show the frequency distribution of E-cadherin and ß-catenin staining in 351 NSCLC specimens. Figure 25 demonstrates the different expression

levels of E-cadherin in squamous cell carcinoma and adenocarcinoma of lung. Figure 26 gives high magnification pictures of Figure 25c and 25f. The pattern of ß-catenin expression resembles that of E-cadherin in NSCLC tumor cells (data not shown).

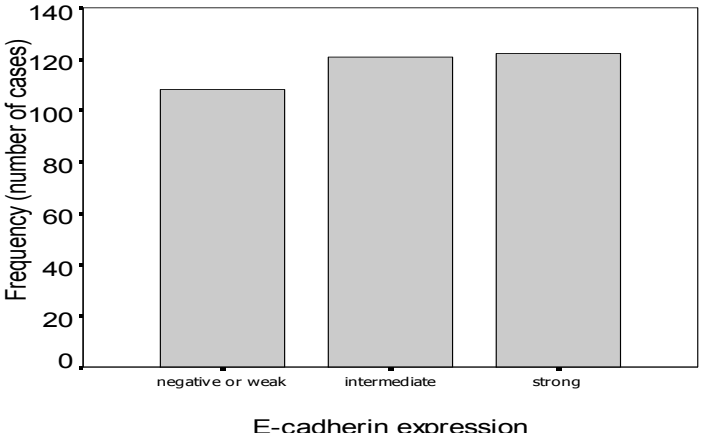


Figure 23. Frequency distribution of E-cadherin staining, *108 cases showed low level expression of E-cadherin,,121 were intermediate and 122 were high level.*

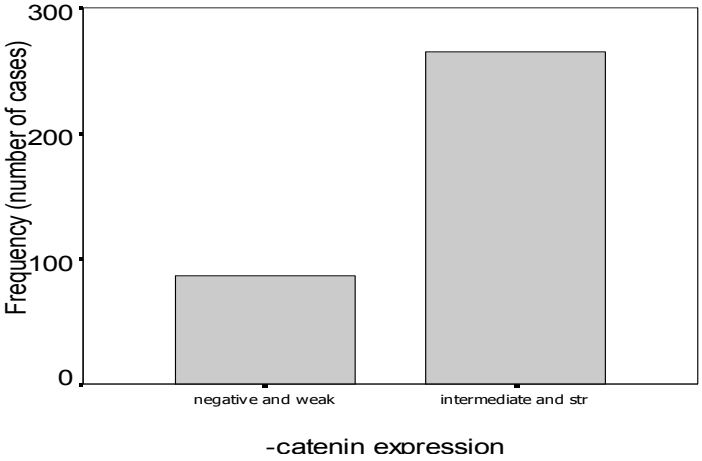


Figure 24. Frequency distribution of β −catenin staining. *86 cases showed low level expression of E-cadherin, and 265 were high level.*

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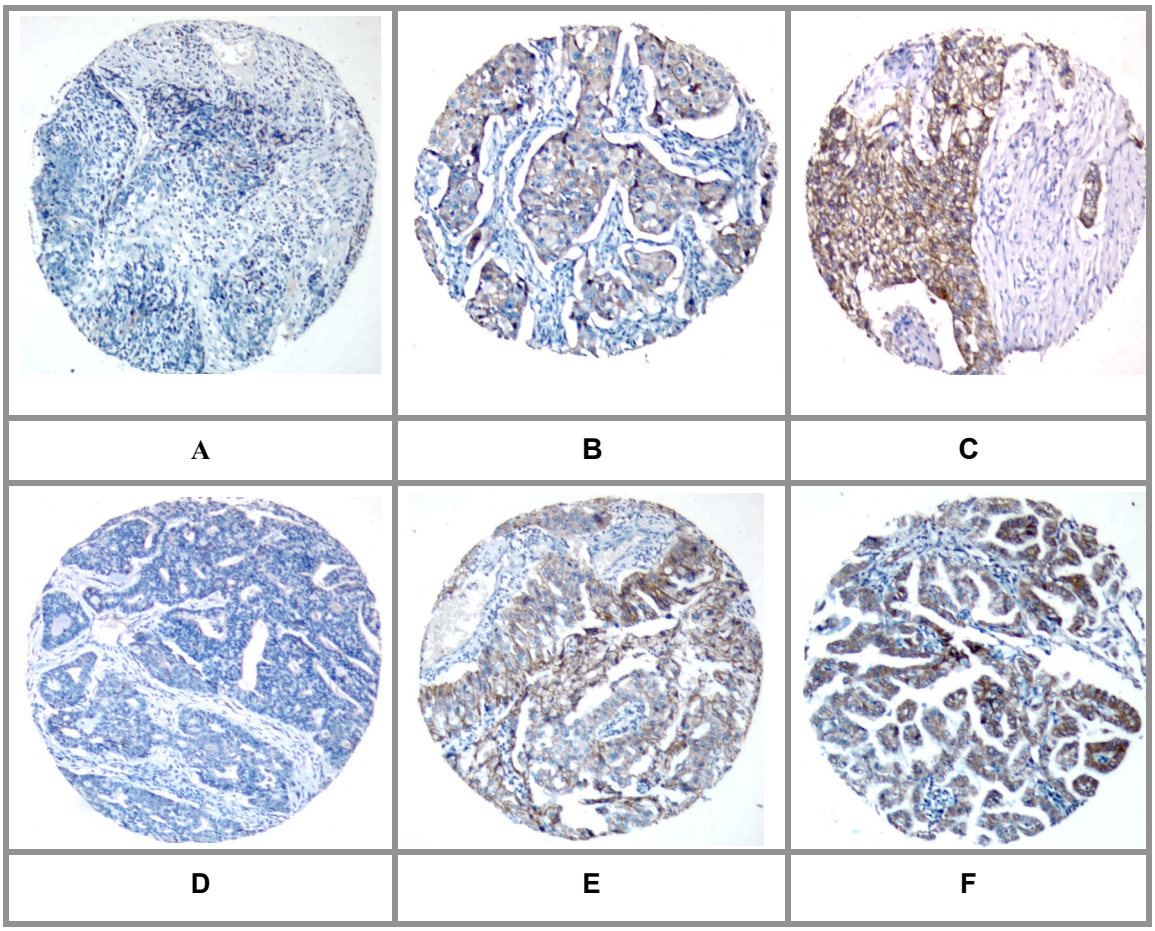


Figure 25. Expression of E-cadherin by immunohistochemical analysis, magnification 1×10. *A, low level expression of E-cadherin in squamous cell carcinoma; B, intermediate expression of E-cadherin in squamous cell carcinoma; C, high level expression of E-cadherin in squamous cell carcinoma; D, low level expression of; E-cadherin in adenocarcinoma. E, intermediate expression of E-cadherin in adenocarcinoma; F, high level expression of E-cadherin in adenocarcinoma*

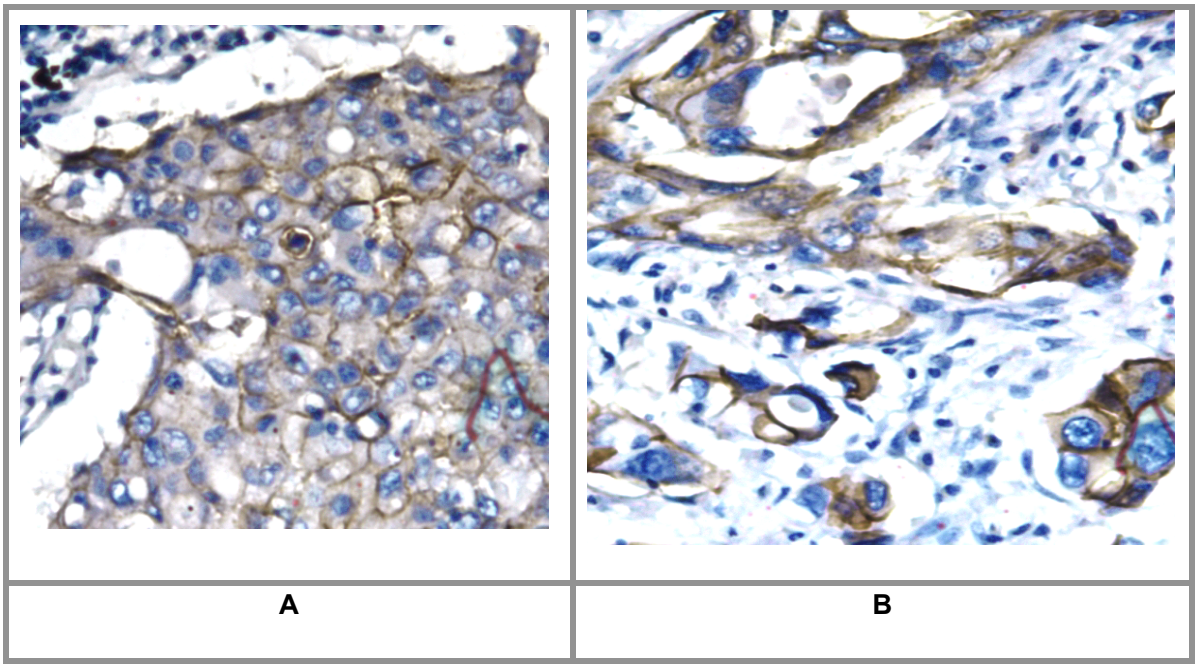


Figure 26. High order magnification of figure 25c*(A)* and 25f *(B)*, *magnification 10×40.*

3.3.7.2 Correlation between E-cadherin and ß-catenin expression and clinicopathological parameters in NSCLC

The expression frequency of E-cadherin in NSCLC is shown in Table 12. One hundred and eight of 351 (30.8%) NSCLC exhibited reduced expression of E-cadherin; including 35 of 113 (31.0%) SCC and 46 of 190 (24.2%) ADC. One hundred and twenty-one cases showed intermediated expression of E-cadherin, and 122 were strong expressors of E-cadherin.

The expression frequency of ß-catenin in NSCLC is shown in Table 13. Eighty-six of 351 (24.5%) NSCLC exhibited low level expression of ß-catenin; including 25 of 113 (22.1%) SCC and 39 of 190 (20.9%) ADC. Two hundred and sixty-five cases showed high level expression of ß-catenin.

There were no statistical differences between E-cadherin expression and age, and metastasis status (M status). The level of E-cadherin expression significantly correlated with tumor status (T status) ($p=0.011$), lymph node status (N status) ($p=0.021$), clinical stage ($p=0.004$) and tumor cell differentiation ($p=0.019$). ß-catenin expression was only related to lymph node status (N status) ($p=0.0210$).

The frequency of low level expression of both E-cadherin and ß-catenin were significantly higher in female than in male ($p=0.019$ and $p=0.009$, respectively). Interestingly, the frequency of low level expression of E-cadherin and ß-catenin was higher in adenosquamous carcinoma than in other cell types ($p<0.001$ and $p=0.001$, respectively).

Parameters	E-cadherin (cases and percentage)			*p*-value
	1+[1]	2+[2]	3+[3]	
Total	108(30.8%)	121(34.5%)	122(34.8%)	
Age				
<59 years	46(25.3%)	66(36.3%)	70(38.5%)	0.295
⩾59 years	62(36.7%)	55(32.5%)	52(30.8%)	
Sex				
Male	72(28.7%)	98(39.0%)	81(32.3%)	0.017
Female	36(36.0%)	23(23.0%)	41(41.0%)	
T status				
1	6(30.0%)	5(25.0%)	9(45.0%)	0.011
2	47(25.0%)	76(40.4%)	65(34.6%)	
3	48(42.9%)	32(28.6%)	32(28.6%)	
4	7(22.6%)	8(25.8%)	16(51.6%)	
N status				
0	40(25.2%)	56(35.2%)	63(39.6%)	0.021
1	22(29.3%)	29(38.7%)	24(32.0%)	
2	42(37.5%)	35(31.3%)	35(31.3%)	
3	4(80.0%)	1(20.0%)	0	
M status				
0	105(31.2%)	121(35.9%)	111(32.9%)	0.051
1	3(21.4%)	0	11(78.6%)	
Stage				
Ⅰ	17(17.3%)	45(45.9%)	36(36.7%)	0.004
Ⅱ	33(35.5%)	29(31.2%)	31(33.3%)	
Ⅲ	55(37.7%)	47(32.2%)	44(30.1%)	
Ⅳ	3(21.4%)	0	11(78.6%)	
Histology				
SCC[4]	35(31.0%)	50(44.2%)	28(24.8%)	<0.001
ADC[5]	46(24.2%)	61(32.1%)	83(43.7%)	
ADSCC[6]	19(47.5%)	10(25.0%)	11(27.5%)	
Others[7]	8(100.0%)	0	0	
Differentiation				
Poor	36(41.4%)	30(34.5%)	21(24.1%)	0.019
Moderate	42(27.5%)	53(34.6%)	58(37.9%)	
Well	30(27.0%)	38(34.2%)	43(38.7%)	

Table 12. Relation between E-cadherin expression and clinicopathological parameters in 351 cases of NSCLC

Abbreviations:[1] *1+ low level expression;* [2] *2+: intermediate expression* [3]*3+: high level expression;* [4]*SCC, squamous cell carcinoma;* [5]*ADC, adenocarcinoma;* [6]*ADSCC, adenosquamous carcinoma;* [7]*others include adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.*

Parameters	β −catenin (cases and percentage)		*p*-value
	1+[1]	2+[2]	
Total	86 (24.5%)	265(75.5%)	
Age			
<59 years	34(20.7%)	130(79.3%)	0.124
⩾59 years	52(27.8%)	135 (72.2%)	
Sex			
Male	52 (20.7%)	199(79.3%)	0.009
Female	34(34.0%)	66(66.0%)	
T status			
1	5(25.0%)	15 (75.0%)	0.385
2	45(23.9%)	143 (76.1%)	
3	31(27.7%)	81 (72.3%)	
4	5 (16.1%)	26 (83.9%)	
N status			
0	33(20.8%)	126(79.2%)	0.021
1	26(34.7%)	49(65.3%)	
2	27(24.1%)	85(75.9%)	
3	0	5(100.0%)	
M status			
0	83(24.6%)	254(75.4%)	0.785
1	3(21.4%)	11(78.6%)	
Stage			
□	16(16.3%)	82(83.7%)	0.061
□	34(36.6%)	59(63.4%)	
□	33(22.6%)	113(77.4%)	
□	3(21.4%)	11(78.6%)	
Histology			
SCC[3]	25(22.1%)	88(77.9%)	0.001
ADC[4]	39(20.5%)	151(79.5%)	
ADSCC[5]	16(40.0%)	24(60.0%)	
Others	6(75.0%)	2(25.0%)	
Differentiation			
Poor	27(24.3%)	84(75.7%)	0.880
Moderate	36(23.5%)	117(76.5%)	
Well	23(26.4%)	64(73.6%)	

Table 13. Relation between β −catenin expression and clinicopathological parameters in 351 cases of NSCLC

Abbreviations:[1] *1+: low level expression,* [2] *2+: high level expression;* [3]*SCC, squamous cell carcinoma;* [4]*ADC, adenocarcinoma;* [5]*ADSCC, adenosquamous carcinoma;* [6]*others include*

adenosquamous cell carcinomas, large cell carcinoma, sarcoma and carcinoid tumor.

3.4 Statistical correlation of markers' expression

In order to evaluate the potential statistical relationship between the different molecular tumor markers examined in this study, the nonparametric Mann-Whitney U test was performed to correlate the expression level of different markers with each other.

Accumulation of p53 was significantly associated with high level expressions of Ki67 (p=0.002), Rad51 (p=0.048) and HER2 (p=0.004) proteins, but not with Ape/Ref-1 expression (Table 14). High level expression of Rad51 was not related to expression of Ki67. By contrast, a significant reverse association was observed between the expression status of Rad51 and that of Ape/Ref-1 (p=0.001). Furthermore, we found an inverse relationship between the expression of Rad51 and HER2 (p=0.005) (Table 15).

Markers	**p53 (cases and percentage)**		*p*-value
	-[1]	+[2]	
Ki67 expression			
-[1]	133 (70.4%)	56 (29.6%)	0.002
+[2]	76 (53.9%)	65 (46.1%)	
total	209 (63.3%)	121 (36.7%)	
Rad51 expression			
-[1]	104 (63.4%)	60 (36.6%)	0.048
+[2]	30 (50.0%)	30 (50.0%)	
total	134 (59.8%)	90 (40.2%)	
HER2 expression			
-[1]	157 (69.5%)	69 (30.5%)	0.004
+[2]	59 (53.6%)	51 (46.4%)	
total	216 (64.3%)	120 (35.7%)	

Table 14. Relationship between expression of p53 and Ki67, Rad51, and HER2 in NSCLC

Abbreviations: [1] *-, low level expression of markers;* [2] *+,high level expression of markers*

Markers	Rad51 (cases and percentage)		*p*-value
	-[1]	+[2]	
Ape/Ref-1 expression			
-[1]	43 (58.9%)	30 (41.1%)	0.001
+[2]	123 (79.9%)	31 (20.1%)	
total	166 (73.1%)	61(26.9%)	
HER2 expression			
-[1]	90 (67.2%)	44 (32.8%)	0.005
+[2]	66 (84.6%)	12 (15.4%)	
total	156 (73.6%)	56 (26.4%)	

Table 15. Relationship between Rad51 and Ape/Ref-1, and HER2 expression in NSCLC

Abbreviations: [1] *-, low level expression of markers;* [2] *+,high level expression of markers*

The results summarizing the relationship between the expression of E-cadherin and ß-catenin in 351 NSCLC tumors are shown in Table 16. Among 86 cases with low level expression of ß-catenin, 78 cases (90.7%) showed low level expression of E-cadherin. Whereas among 265 cases with high level expression of ß-catenin, just 30 (11.3%) showed low level expression of E-cadherin. The correlation between the levels of E-cadherin and ß-catenin expression was statistically significant ($P < 0.001$).

E-cadherin expression	β-catenin expression		*p*-value
	Low level expression	High level expression	
Low level expression	78(90.7%)	30(11.3%)	<0.001
Intermediate expression	4(4.7%)	117(43.4%)	
High level expression	4(4.7%)	118(43.8%)	
Total	86(100%)	265(100%)	

Table 16. Relationship between E-cadherin and ß-catenin expression in NSCLC

3.5 Relationship between marker expression and clinical outcome in NSCLC: univariate survival analysis

The results of marker expression were analyzed with regard to overall survival time of the patients. Univariate survival analysis (log-rank test) demonstrated significant association between overall survival and the status of Ape/Ref-1 (p=0.0397), E-cadherin (p<0.0001) and ß-catenin (p=0.0067). Patients with low-level expression of Ape/Ref-1, E-cadherin, and ß-catenin had a poorer prognosis than did those with high-level expression. There was no significant relation between the expression status of p53, Ki67, HER2, or Rad51 and overall survival. Table 17 presents the results of univariate analysis with regard to markers expression and overall survival of NSCLC. Kaplan-Meier survival curves are not shown here.

Although the differences did not reach the statistical significance with regard to the expression of Rad51 (p=0.060), the lung cancer-related 5-year overall survival probabilities were 49% in the low level expression group compared to 33% in the high level expression group. Median survival time was 51 months for patients with low expression of Rad51, but 25 months for patients with high level expression of Rad51.

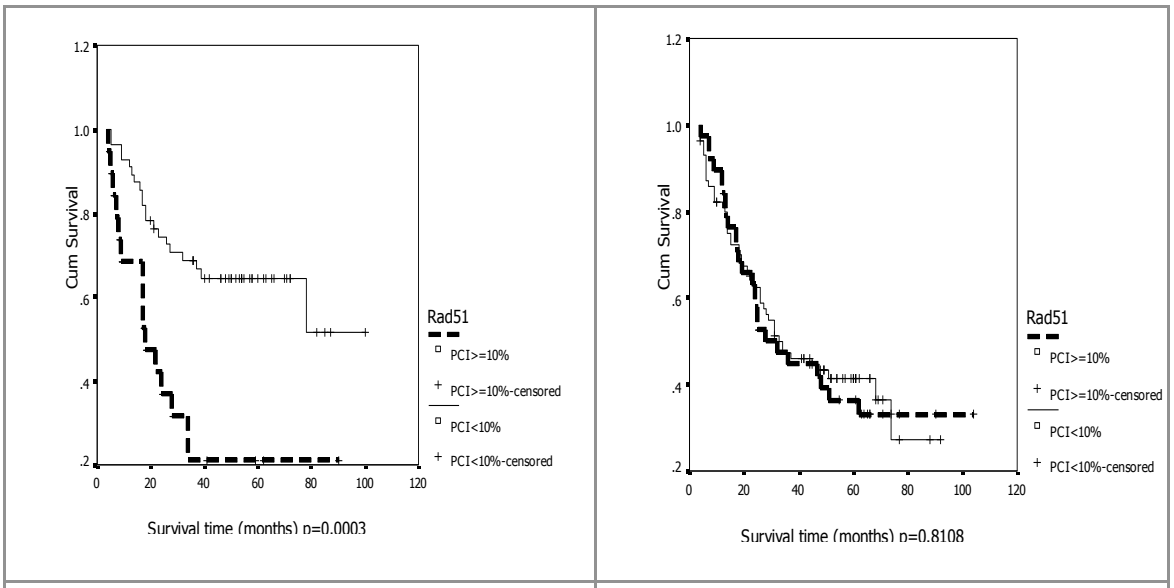
Additionally, survival analysis was performed with regard to marker expression in subsets of patients with different histological cell types. Figure 27 to 32 show Kaplan-Meier survival curves in relation to tested markers in subsets of patients with squamous cell carcinoma and adenocarcinoma of the lung. The Stratified survival analysis by tumor cell type revealed that expression of Ape/Ref-1 and E-cadherin had an important impact on lung cancer survival regardless of the histological cell type, Patients with low-level expression of Ape/Ref-1 and E-cadherin had a poorer prognostic outcome regardless of squamous cell carcinoma and adnocarcinoma of NSCLC (Figure 28 and Figure 31, respectively). The expression of p53 had significant prognostic value in adenocarcinoma (p=0.0400), but not in squamous cell carcinoma, high level

expression of p53 was a poor prognostic factor for adenocarcinoma, but not for squamous cell carcinoma of the lung (Figure 29). Rad51, HER2 and ß-catenin expression were closely related to the survival time of patients suffering from lung squamous cell carcinoma (p=0.0003, p=0.0010 and p=0.0317, respectively), but not related to that of lung adenocarcinoma (Figure 27, Figure 30 and Figure 32, respectively). High level expression of Rad51 and HER2 closely associated with short survival time of patients with lung squamous cell carcinoma. Low level expression of ß-catenin was a poor prognostic factor for patients with squamous cell carcinoma of the lung.

There was no significant difference between the expression of Ki67 and overall survival time of lung cancer. Moreover, stratified survival analysis by tumor cell types could not reveal any correlation between Ki67 expression and survival of either squamous cell carcinoma or adenocarcinoma (Kaplan-Meier survival curves are not shown)

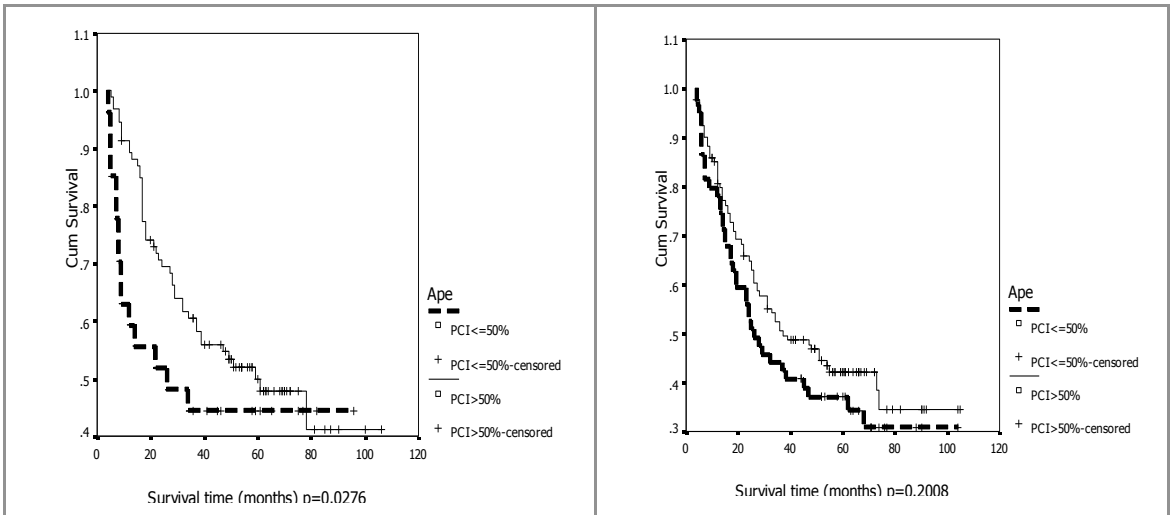
Markers	Patients (cases)	Mean survival (months)	Median survival (months)	5-Year Survival (%)	*p*-value
Rad51					
Low level expression	170	55	51	49	0.060
High level expression	65	47	25	33	
Ape/Ref-1					
Low level expression	100	49	26	38	0.040
High level expression	248	58	49	47	
P53					
Low level expression	227	57	45	45	0.416
High level expression	133	50	31	43	
Ki67					
Low level expression	197	56	46	45	0.554
High level expression	144	54	31	42	
HER2					
Low level expression	231	55	42	45	0.839
High level expression	116	55	33	43	
E-cadherin					
Low level expression	108	30	16	19	<0.001
Intermediate expression	121	50	33	42	
High level expression	122	66	77	54	
ß-catenin					
Low level expression	86	41	24	31	0.007
High level expression	265	55	36	42	

Table 17. Predictive markers for prognosis of NSCLC patients by univariate analysis (log-rank test)



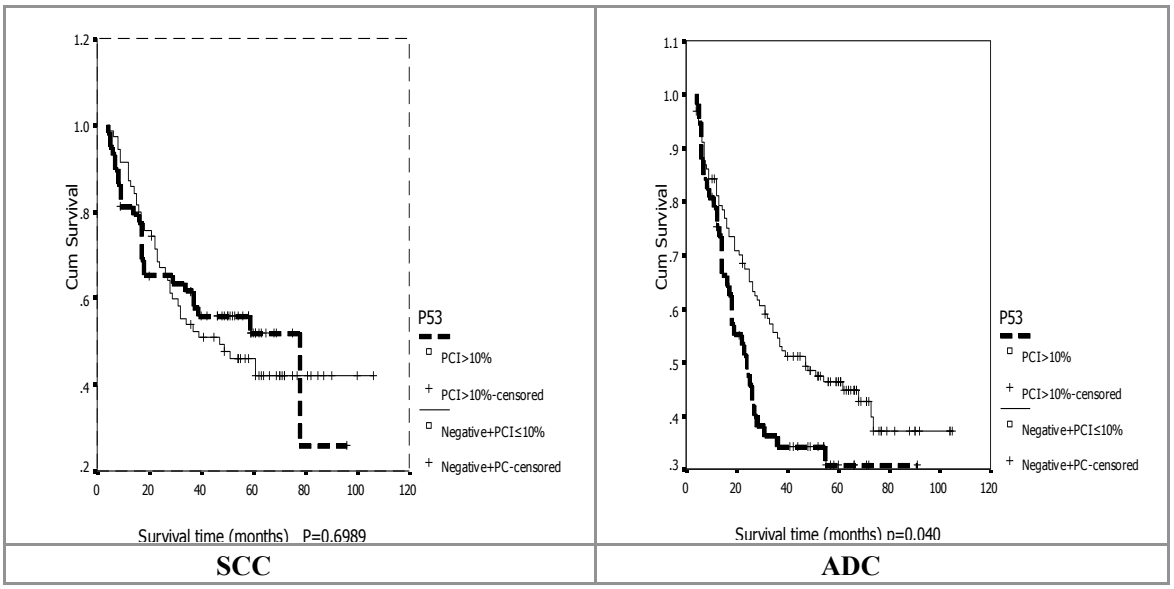
SCC	ADC

Figure 27.. **Kaplan-Meier survival analysis according to Rad51 expression (log-rank test)***:* **SCC***, probability of survival of squamous cell carcinoma of NSCLC patients: low level expression (solid line), n=55; high level expression (dashed line), n=19.* **ADC***, probability of survival of adenocarcinoma of NSCLC patients: low level expression (solid line), n=85; high level expression (dashed line), n=38.*



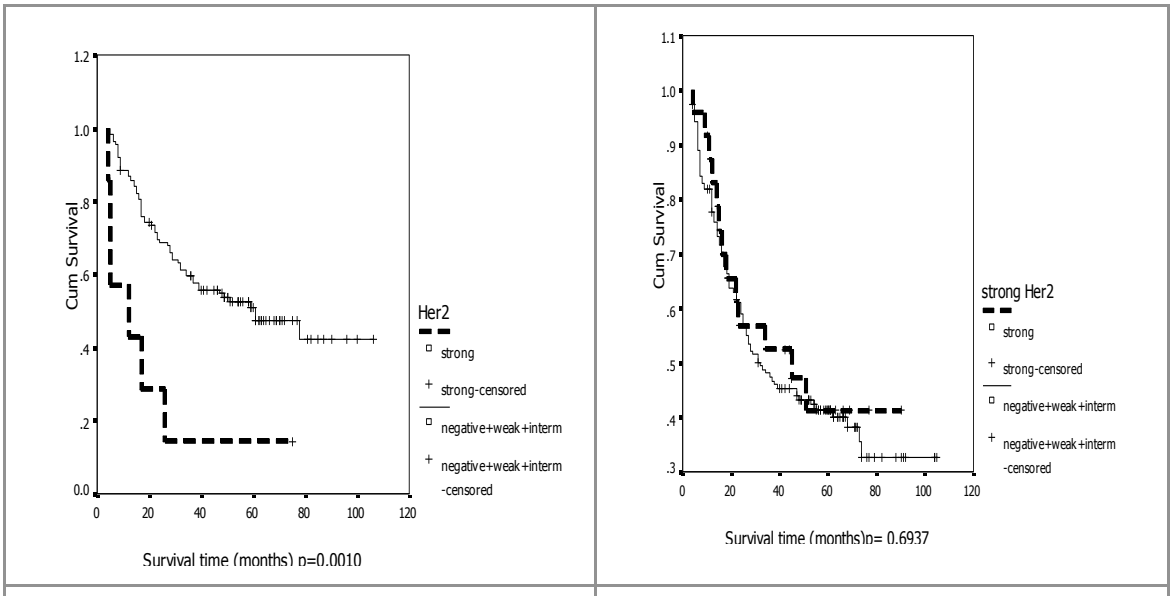
SCC	ADC

Figure 28. Kaplan-Meier survival analysis according to Ape/Ref-1 expression (log-rank test)*:* **SCC***, probability of survival of squamous cell carcinoma of NSCLC patients: low level expression (dashed line), n=27; high level expression (solid line), n=93.* **ADC***, probability of survival of adenocarcinoma of NSCLC patients: low level expression (dashed line), n=59; high level expression , n=120.*



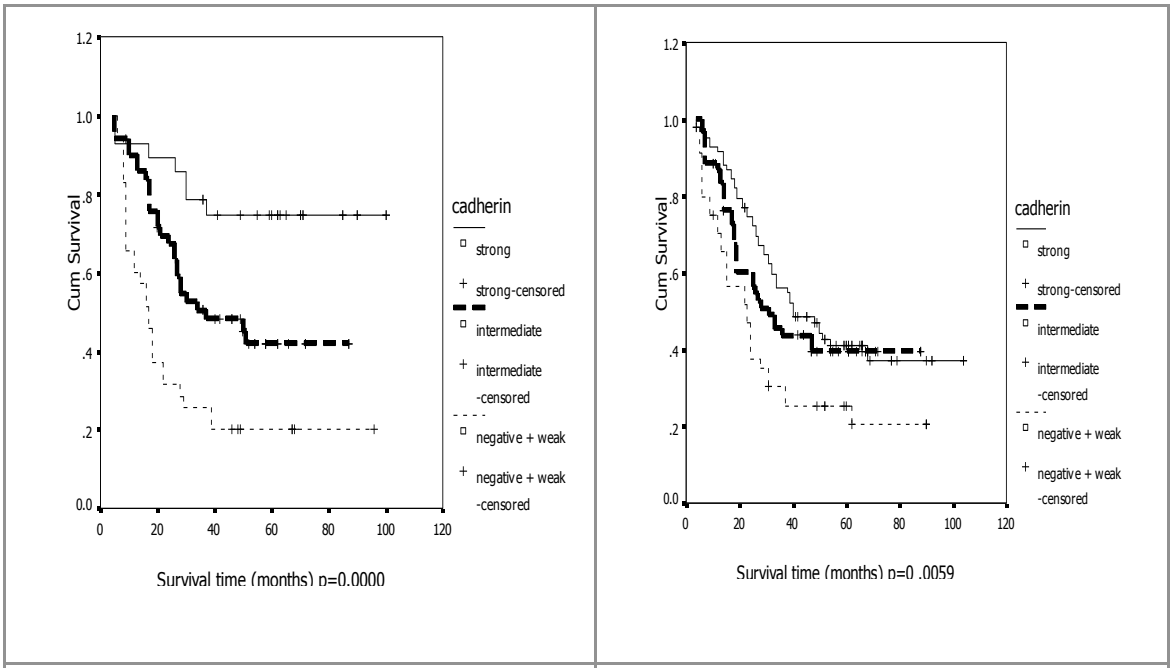
SCC	ADC

Figure 29. Kaplan-Meier survival analysis according to p53 expression (log-rank test): **SCC**, *probability of survival of squamous cell carcinoma of NSCLC patients: low level expression (solid line), n=70; high level expression (dashed line), n=58.* **ADC**, *probability of survival of adenocarcinoma of NSCLC patients: low level expression (solid line), n=122; high level expression (dashed line), n=57.*



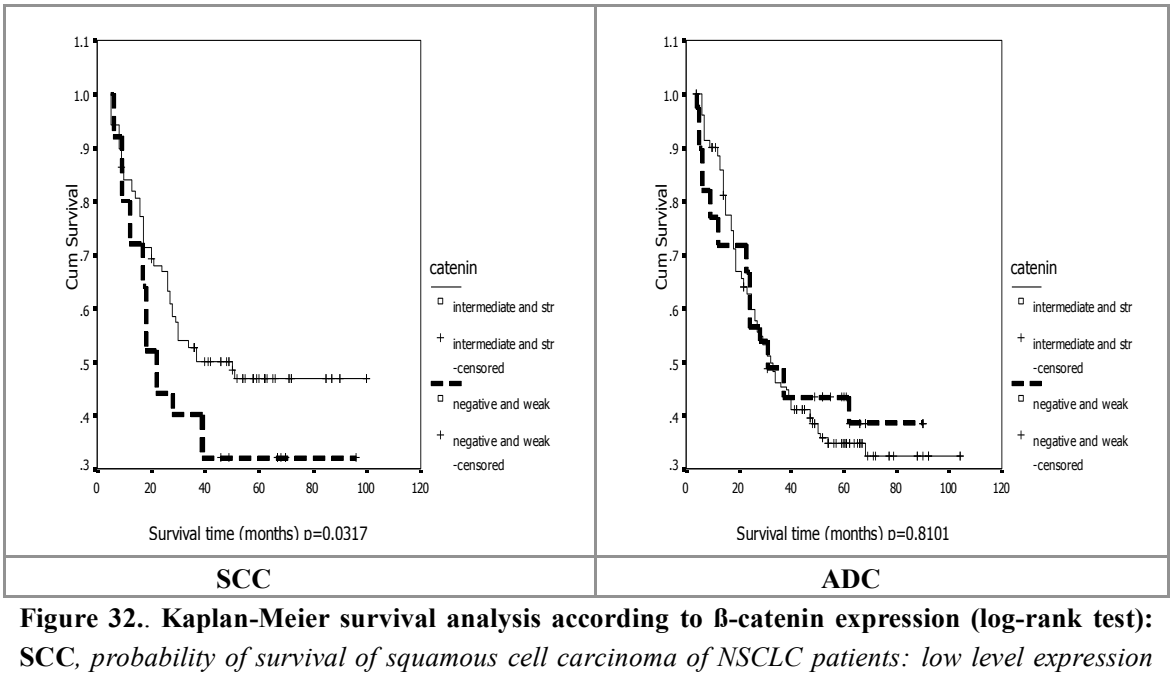
SCC	ADC

Figure 30. Kaplan-Meier survival analysis according to HER2 expression (log-rank test): **SCC**, *probability of survival of squamous cell carcinoma of NSCLC patients: low level expression (solid line), n=113; high level expression (dashed line), n=7.* **ADC**, *probability of survival of adenocarcinoma of NSCLC patients: low level expression (solid line), n=154; high level expression (dashed line), n=24.*



SCC	ADC

Figure 31. Kaplan-Meier survival analysis according to E-cadherin expression (log-rank test): **SCC**, *probability of survival of squamous cell carcinoma of NSCLC patients: low level expression (dotted line), n=35; intermediate expression (dashed line), n=50, high level expression (solid line), n=28.* **ADC**, *probability of survival of adenocarcinoma of NSCLC patients: low level expression (dotted line), n=46; intermediate expression (dashed line), n=61, high level expression (solid line), n=83*



SCC	ADC

Figure 32.. Kaplan-Meier survival analysis according to ß-catenin expression (log-rank test): SCC, *probability of survival of squamous cell carcinoma of NSCLC patients: low level expression (dashed line), n=25; high level expression (solid line), n=85.* **ADC**, *probability of survival of adenocarcinoma of NSCLC patients: low level expression (dashed line), n=39; high level expression (solid line), n=151.*

3.6 Independent prognostic factors of NSCLC: Cox multiple regression analysis

In order to eliminate synergetic effects on overall survival between different significant factors in univariate analysis, a multivariate Cox regression analysis was carried out. Since variables found to have prognostic influence by univariate analysis may covariate, all statistically significant variables from the univariate analysis were included in the multiple regression analysis to identify independent prognostic factors.

Primary tumor size (T), lymph node metastasis (N), distant metastasis (M), clinical stage, tumor cell differentiation, expression of E-cadherin and Ape/Ref-1 were included in the multiple regression analysis.

Data from the multivariate analyses are presented in Table 18. E-cadherin expression proved to be an independent prognostic factor ($p < 0.001$) as did lymph node metastasis ($P =< 0.040$), clinical stage ($P < 0.001$), and differentiation ($P < 0.001$).

Factors	Beta	Standard error	Wald	df	Relative risk(RR)	95% CI	*p*-value
N status[1]	0.197	0.112	3.063	1	1.217	0.977-1.517	0.040
Stage[2]	0.472	0.117	16.179	1	1.603	1.274-2.018	<0.001
E-cadherin[3]	0.542	0.089	37.408	1	1.720	1.445-2.046	<0.001
Differentiation[4]	0.376	0.099	14.308	1	1.456	1.198-1.769	<0.001

Table 18. Multivariate analysis on overall survival (Cox proportional hazard model)

[1] N_3 *vs* N_2 *vs* N_1 *vs* N_0

[2] □ *vs* □ *vs* □ *vs* □

[3] Negative or weak *vs* intermediate *vs* strong

[4] Poor *vs* moderate *vs* well

Furethermore, multivariate Cox regression analyses were carried out according to subsets of tumor cell types. For squamous cell carcinoma, T status, N status, M status,

stage, differentiation, expression of E-cadherin, Ape/Ref-1, Rad51, HER2 and ß-catenin were included in the multiple regression analysis. For adenocarcinoma, T status, N status, M status, stage, differentiation and expression of p53 were included in the multiple regression analysis. None of the expression of Ape/Ref-1, Rad51, p53, HER2 and ß-catenin was shown as independent prognostic factors for squamous cell carcinoma or adenocarcinoma of the lung.

4 Discussion

4.1 Significance of molecular prognostic studies of NSCLC

Non-small cell lung cancer (NSCLC) is best conceptualized as a group of heterogeneous clinical entities that share the same cellular origins but that have different clinical behaviors, and hence, different prognoses. Determining the prognosis for an individual patient with NSCLC is difficult. Thus, prognostic factors are used to divide the population into subgroups in order to distinguish groups of patients with different outcome [Feinstein, 1972] and chose the appropriate treatment for the individual patient.

Up to date, the TNM staging system is considered the most accurate predictive factor for NSCLC. However, the anatomic TNM staging system, based on histopathology and extent of disease at presentation, has reached its limit in providing critical information that may influence the strategy of treatment. Patients with the same pathologic and clinical stage of NSCLC display considerable variability in recurrence and survival. A significant proportion may suffer from regional or distant recurrence after “standard treatment”. Consequently, there is an urgent need for new prognostic and predictive molecular markers, which can distinguish between patients with unfavorable prognosis and others with favorable prognosis

A substantial amount of research on the biology of NSCLC has focused on the evaluation of new molecular markers present in tumor cells as prognostic factors or possible targets for therapy. As a consequence, with the exception of a few predictive factors, the literature demonstrates conflicting evidence of the prognostic power of each factor. For a small number of factors, studies are largely consistent in their findings. For example, in the great majority of studies, at least one representation of prognostic TNM stage has been demonstrated as being significantly related to prognosis [Grondin and

Liptay, 2002]. In contrast, three factors (*e.g.*, tumor cell type, patient sex, and patient age) have consistently been found not to be significantly associated with survival outcomes [Michael et al., 2002]. For many other factors, the strength of the independent association of that factor with survival outcomes is also quite variable. There are many potential reasons for this observed heterogeneity. Some studies are clearly statistically underpowered, given that the median number of patients enrolled per study was only around 100 (the least number was 31) [Hashimoto et al., 2000]. Variation in the methods used to define and quantify prognostic factors will be reflected in the heterogeneity of the apparent strength of association between the prognostic factor and the relevant outcome.

As introduced above, despite a variety of molecular markers have been implicated in the prognosis of NSCLC, conflicting results were widely reported in the literature. Thus further investigations will be required, especially the use of high throughput tissue microarrays and the development of appropriate markers or panels of molecular markers, as well as good quality control in detection [Niklinski et al., 2001].

4.2 Application of the tissue microarray technique in molecular prognostic study

The tissue microarray (TMA) technique was firstly described by Kononen from the NIH, U.S.A. in 1998 [Kononen et al., 1998]. This technique, which can facilitate research on a large series of tissues in parallel in a single experiment, has been widely used in cancer research and other fields. During the designing period of the present study, there were only few studies using TMA in molecular prognostic research [Richter et al., 2000]. Meanwhile, there are hundreds of papers on molecular prognostic studies using TMA. The major advantages of TMA technique in molecular studies can be summarized as follows:

- Economic and speedy: The conventional investigation of fresh frozen or paraffin

embedded tissues is too expensive and time consuming to be applied to the characterization of hundreds or thousands of genes associated with distinct tumor entities. TMA places a large scale of tissue samples on one slide and in a single experiment. Thus, only a very small amount of reagent is required to analyze a cohort of specimens, and hundreds of experiments can be carried out. It not only saves money but also time [Kononen et al., 1998].

- Reliable and comparable: The most important advantage of TMA used in prognostic studies is its experimental uniformity. Using this technique, each tissue sample on the same TMA slide is treated in an identical manner. It allows the entire cohort to be analyzed in one batch on a single slide. Thus reagent concentrations are identical for each case, as are incubation times and temperatures, washing conditions, antigen retrieval, etc. TMA derived results are more reliable and comparable than those from conventional methods, since conventional sections can have substantial slide to slide variability associated with processing (for example, 20 batches of 15 slides to stain 300 slides).

On the other hand, a commonly expressed concern about the usefulness of TMA analysis is whether the small core samples (0.6 mm in diameter) give meaningful information on large tumor specimens. Three studies have directly compared biomarker expression using TMAs and regular sections of the same breast cancers. All studies report >90-95% concordance for common breast cancer biomarkers such as estrogen and progesterone receptors and the HER-2 oncoprotein [Bucher et al., 1999; Gillett et al., 2000; Camp et al., 2000]. Moreover, prognostic associations for these markers could be reproduced with the TMAs [Bucher, et al., 1999]. Some investigators have used core samples that are larger in diameter ($\geq$2-4 mm) to improve the representativity. However, larger core samples did not substantially increase the information content of TMA analysis, since the likelihood of finding heterogeneity within such a small area is often quite low [Kallioniemi et al., 2001].

Theoretically, TMA analysis is fundamentally different from conventional histological

analyses. This technology is a population-level research tool. It is not intended for making clinical diagnoses of individual cases. Obviously, samples measuring 0.6 mm in diameter will not capture all the information from large, sometimes highly heterogeneous tumors. Analysis of molecular markers on TMAs may therefore result in lower prevalence estimates than obtained from conventional tumor sections. This will depend on the degree of heterogeneity of the examined tumor type and the marker. Molecular targets that have prognostic significance are often relatively uniformly expressed in cancer tissues. Sampling methods used for TMAs are therefore suitable for detecting such critically important promising prognostic markers.

These advantages encouraged us to design the present study for investigating the prognostic value of DNA repair factors Rad51 and Ape/Ref-1, as well as p53, Ki67, HER2, E-cadherin and ß-catenin in NSCLC using the TMA technique.

4.3 Representation and reliability of this study

In order to avoid case selective bias in our clinical retrospective prognostic study, all 383 NSCLC patients were of Chinese race, consecutively recruited in one single hospital. The Lung Cancer Research Institute of Guangdong province, China, is the largest lung cancer special medical center in southern China, and more than 30% of total lung cancer patients in this area are registered and treated in this institute. All of the patients were recruited according to pre-established inclusion criteria. Demographic and clinico-pathological analyses demonstrated that distribution of age, sex and histological cell type were consistent with the general situation of NSCLC in the local area.

As discussed above, in order to improve the comparability and reliability of the study, we used the TMA technique in combination with immunohistochemistry (IHC). Quality control in IHC is one of the major problems in daily practice. Even though IHC has been in use now for decades, there is still a high variability of intra-laboratory and inter-laboratory results, due to differences in antigen retrieval, staining protocols,

antibodies used, and in the interpretation of staining results. The use of TMAs solves many of these quality control problems [Packeisen et al., 2003]. In order to avoid experimental bias, we set 4 internal negative controls (one normal lung tissue and 3 tissues of benign diseases of lung) on the TMAs, and in addition, an external negative control at every experiment. IHC for a specific antibody was repeated at least in two independent experiments. Cases whose IHC results were not in accordance with each other in different experiments were deleted. Furthermore, the assessment of IHC was performed without knowledge of clinico-pathological data. All of these procedures guaranteed the reliability of our results.

4.4 Determination of Cut-off point in this study

Variation in classifying of the factors analyzed is one of the reasons that lead to conflicting results in existing reports. In this sense, determination of the cut-off point of immunohistochemistry, which can classify the expression of the marker into different prognostic groups, is a very important issue in molecular prognostic study [Lee et al., 1995].

Although the selection of the median or mean value of the expression could be a standard approach to analyze new prognostic factors, it usually cannot be used to divide patients into significant prognostic groups. The choice of cut-off points has often been arbitrary in previous studies with the result that authors assessed their respective IHC data in different ways. Up to now, no standard and unified approach for determination of cut-off point has been published.

As a rule, cut-off point should be determined beforehand according to the distribution frequency of immunohistochemical staining data, and then correlated with survival data to find out which point value could separate best patients into different outcome groups.

In this study, we initially counted IHC stained tumor cells and analyzed the distribution of

positively stained tumor cells and negative tumor cells in the whole cohort of tumors. Subsequently, we set several candidate values for cut-off points around mean or median values, and tentatively calculated and evaluated the prognostic role of the specific marker according to the different candidate cut-off point values. Finally, an optimized cut-off point was determined and patients were classified into two different categories according to the expression profile of specific marker.

The only exception to this rule was the expression of E-cadherin. Here we divided E-cadherin expression into three groups (□low level expression, □intermediate expression and □high level expression), since two cut-off points could separate best patients into different outcome groups.

4.5 DNA repair and prognosis of NSCLC

Alteration of DNA repair mechanisms is well recognized as a pathogenetic event in cancer progression [Loeb, 2000]. More and more evidence suggests that DNA repair is involved in the development of the tumor resistance towards cytotoxic agents and irradiation. Thus, we hypothesized that DNA repair capability may play a role in the prognosis of NSCLC. In the present study, we evaluate two major DNA repair factors: homologous recombination factor Rad51 and base excision repair factor Ape/Ref-1.

4.5.1 Rad51 and NSCLC

Rad51 is the key enzyme for homologous recombination, an evolutionarily conserved mechanism for the repair of DNA damage and the generation of genetic diversity. In order to investigate the role of Rad51 in NSCLC, we immunohistochemically detected Rad51 expression in NSCLC tissues. Consistent with studies in several tumors, including breast cancer, pancreatic adenocarcinoma and fibroblasts, etc [Maacke et al., 2000a; Maacke et al., 2000b; Raderschall et al., 2002b], our results demonstrate that Rad51 is over-expressed in tumor cell nuclei in NSCLC compared to normal tissues. 27.7% NSCLC tumors showed high level expression of Rad51. These findings, together

with results from previous studies, suggest that abnormal expression of the mammalian Rad51 recombination could play a major role in the multistep process of tumorigenesis

Most importantly, the prognostic role of Rad51 for NSCLC was evaluated in this study. To our knowledge, this is the first study analyzing the relationship between Rad51 expression and the prognosis of NSCLC. Although it did not reach statistical significance ($p=0.060$), our study revealed that the median survival time and 5 year survival rate in patients with high-level expression of Rad51 appears shorter and lower than that with low Rad51 expression. The median survival time in patients with Rad51 high level expression was 25 months, compared to 51 months for patients with low level expression of Rad51. Five-year survival rate of patients with Rad51 high-level expression was 33%, but for patients with low-level expression of Rad51 it was 49%. Additionally, stratified survival analysis by histological tumor cell type demonstrated that Rad51 expression was closely related to the survival of lung squamous cell carcinoma, but not related to that of lung adenocarcinoma and other types. Therefore, our findings demonstrate that Rad51 expression has the potential to predict the outcome of squamous cell lung cancer. The assessment of Rad51 expression may therefore be used as an additional tool in identifying those patients at risk of tumor recurrence and progression, and it may be a helpful criterion to optimize individual therapy management.

Our finding that Rad51 expression was closely related to the survival of lung squamous cell carcinoma but not adenocarcinoma may indicate that different cellular mechanisms are responsible for the progression of squamous cell carcinoma and adenocarcinoma of the lung. Recent evidence demonstrate that substantial genomic differences between squamous carcinomas and adenocarcinomas. For example, p53 alteration is more frequent in squamous cell carcinoma than in adenocarcinoma, whereas Ras alteration is more prevalence in adenocarcinoma than in squamous cell carcinoma of lung [Massion and Carbonehe, 2003; Petersen et al., 1997]. Thus, it is rational that significant differences in the total number of abnormalities between squamous

carcinomas and adenocarcinomas could result in the differences in the level of genome instability and/or in the mechanisms by which they progress, and in turn lead to different prognostic role of the specific markers for squamous carcinomas and adenocarcinomas.

Our findings that Rad51 expression was elevated in tumor cells of NSCLC and elevated expression of Rad51 was related to shorter survival time of patients with squamous cell lung carcinomas raise the question of the biological function of Rad51 again. A report indicated that elimination of Rad51 in mice leads to sensitivity towards ionizing radiation and early embryonic lethality [Tsuzuki et al., 1996]. Conditional inhibition of Rad51 transcription in untreated chicken DT40 cells results in G_2/M arrest with high levels of chromosome-type breaks [Sonoda et al., 1998]. These data suggested that Rad51 might be essential for survival in higher eukaryotic cells, presumably due to its role in repairing DNA double-strand breaks arising during DNA replication [Rassool et al., 2003]. Moreover, there is evidence of elevated expression of Rad51 and/or homologous recombination activity in immortal human cells, which may contribute to survival and/or increased genetic instability [Xia et al., 1997]. Enhanced expression of Rad51 protein in tumor cells is associated with high DNA repair capacity, elevated recombination rates and increased resistance against radio- and chemotherapy [Maacke et al., 2000a; Vispe S et al., 1998; Raderschall E et al., 2002a]. Our present results of NSCLC and results of papillary bladder carcinoma (Krüger and Stürzbecher, data not shown), as well as breast cancer [Maacke et al., 2000b] and pancreatic cancer [Maacke et al., 2000a] strongly suggest that high-level Rad51 expression might be a permissive event for tumor progression, probably because it will help to keep DNA damage at a tolerable level for cell survival and at the same time will enhance genetic instability. Unfortunately, no adequate information on adjuvant treatment of patients was available in this retrospective series. So, no conclusion can be drawn about a potential role of Rad51 expression as predictor of response to therapy. This hypothesis is however very tempting on the basis of *in vitro* data indicating the Rad51 activity in mechanisms of

repair after the insult of DNA damaging agents or irradiation, and our result indicating Rad51 potential for prediction of prognosis of lung squamous cell carcinoma.

The evidence that in a variety of cancers - pancreas and mammary carcinomas, skin cancers and as shown here in NSCLC - only a subset of tumor cells show high-level expression of Rad51 suggests that abnormal expression of Rad51 in malignant tumors might be regulated by epigenetic mechanisms. Therefore, the regulation of Rad51 expression and interaction between altered Rad51 and other proteins, including normal and abnormal status, are very important issues in this area. Although tumor suppressor proteins such as p53 and Brca2 interact with Rad51 directly and are thought to keep Rad51 in an inactive monomeric state [Stürzbecher et al., 1996; Sharan et al., 1997], the exact mechanism of interaction between Rad51 and altered p53 protein remains unclear. Magnusson and coworker reported that the lack of sufficient levels of wild-type p53 and increased levels of HsRad51 protein coexist in fibroblasts, and may contribute to the elevated RecA-like activity [Magnusson et al., 2000]. Our results showed that the relationship between high level expression of Rad51 and accumulation of p53 was statistical significance. In addition, in the present study, statistical analyses revealed an inverse relationship between Rad51 and HER2 expression levels in NSCLC. Although it is difficult to understand, our results indicating relationship between high level expression of Rad51 and altered p53 and HER2 proteins imply that the regulations and posttranscriptional modification of Rad51 are very complicated mechanisms, and further studies need to be conducted to explain these phenomena.

In conclusion, the present study revealed elevated expression of Rad51 in NSCLC tumors. Moreover, the poor prognostic role of elevated Rad51 in patients with lung squamous cell carcinoma may be due to enhanced homologous recombination and its related tumor resistance against chimo- or radio-therapy. Our findings have possible therapeutic applications. Firstly, Rad51 could serve as a prognostic marker to improve tumor classification. More importantly, down-regulation of Rad51 protein by Rad51 antisense oligonucleotidesor Rad51-inhibitory drugs could be used to sensitize tumors

to radiation or chemotherapy.

4.5.2 Ape/Ref-1 and NSCLC

AP sites are possibly the most common DNA lesions and may occur spontaneously or during the repair of modified bases. Base excision repair of AP sites requires multiple steps, beginning with the cleavage of the DNA strand adjacent to AP sites. DNA strand cleavage is catalyzed by an APE (APE/ref-1) and occurs 5' to the AP site [Demple et al., 1994]. APE/ref-1 is believed to be the rate-limiting step in the base excision repair pathway. Besides the activity in DNA repair, Ape/Ref-1 is involved in the redox regulation of transcription factors by stimulating the DNA binding activity of various proto-oncogene products such as c-Jun, c-Fos, c-Myb [Puglisi ET AL., 2002]. Numerous investigations have shown the presence of increased Ape/Ref-1 in a variety of malignancies, including colon, ovarian, ovarian, cervical, germ cell, and prostate. [Xu et al., 1997; Moore et al., 2000; Kakolyris et al., 1997]. A number of investigations have examined the role of Ape/Ref-1 in carcinogenesis by over-expression and antisense studies. Knockout of the mouse homolog of Ape/Ref-1 leads to embryonic lethality [Xanthoudakis et al., 1996]. Antisense studies show that decreasing the level of Ape/Ref-1 sensitizes cells towards numerous toxic agents [Ono et al., 1994]. Robertson found decreased Ape/Ref-1 appears to be related to the induction of apoptosis [Robertson, 1997]. These findings suggested that Ape/Ref-1 might be involved in some aspects of carcinogenesis and the progression of cancers.

In the present study, we have examined the immunohistochemical expression of Ape/Ref-1 in NSCLC. Our results demonstrated that the predominant pattern of Ape/Ref-1 expression in this series of NSCLC was nuclear. Some of the tumor cells showed cytoplasmic staining or both nuclear and cytoplasmic staining. The different localization of Ape/Ref-1 in NSCLC cells suggests a variety of roles for this multifunctional protein. In addition, we found that nuclear high level expression of Ape/Ref-1 was related to histological tumor cell differentiation, but not to other

clinico-pathological features. The frequency of high level expression of nuclear Ape/Ref-1 was significantly higher in well differentiated tumor cells than that in moderately and poorly differentiated tumor cells. This finding, in agreement with Tell's report [Tell et al., 1999], suggests that elevated nuclear expression of Ape/Ref-1 seems related to tumor cell differentiation.

Tumors are known to be hypoxic and lung cancers usually arise in the context of severely affected lung tissue (smokers, fibrotic lung, abestosis, etc), suffering from a hypoxic challenge [Moulder et al., 1984]. Previous studies have demonstrated the induction of high level expression of Ape/Ref-1 in hypoxic conditions [Walker et al., 1994; Yao KS et al., 1994]. Therefore, high level expression of Ape/Ref-1 might either be required for the correction of DNA damage caused by the re-oxygenation of cells in a hypoxic environment, or Ape/Ref-1 might have a repair function in mitochondrial DNA. Mitochondria have been reported to possess AP endonucleases and mitochondrial DNA is considered to be more susceptible to hypoxia, resulting in a higher rate of DNA errors [Driggers et al., 1996]. It is not surprising that APE/Ref-1 has been shown to be an important regulator of p53 function through both redox-independent and -dependent means [Meira et al., 1997]. Their findings, along with the fact that hypoxic stresses induce the accumulation of both APE/ref-1 and p53, suggest plausible links between oxidative damage, the activation of DNA repair and redox transcriptional functions, and consequent influences on cellular proliferation and apoptotic processes. In a clinical study, Kakolyris has found an inverse association between nuclear expression of Ape/Ref-1 and p53 expression in squamous lung carcinomas [Kakolyris et al., 1999]. In contrast, we could not find a statistical relationship between nuclear expression of Ape/Ref-1 and p53 expression either in squamous cell carcinoma or in adenocarcinoma. Interestingly, a statistical inverse relationship between nuclear expression Ape/Ref-1 and Rad51 expression was observed in our study. This finding implies that Ape/Ref-1 has broad interaction with functional genes and proteins, but the relationship between Ape/Ref-1 and Rad51 is not known presently.

A prognostic role of Ape/Ref-1 was described in a series of early operable NSCLC [Kakolyris et al., 1999]. The authors reported a survival benefit for patient whose tumors expressed high level nuclear Ape/Ref-1. Our result is consistent with their report. We found that high level nuclear expression of Ape/Ref-1 is a favorable factor for NSCLC in univariate analysis regardless of the tumor cell type. These observations are in contrast to a recent study in breast cancer which demonstrated a survival disadvantage for patients whose tumors showed Ape/Ref-1 staining in both nuclear and cytoplasm [Puglisi et al., 2002]. Our results did not show any relationship between subcellular expression of Ape/Ref-1 and clinico-pathological features, survival of patients, and other markers (e.g., p53). A recent investigation revealed that elevated Ape/Ref-1 expression in human testicular cancer may be related to their relative resistance to therapy [Robertson et al., 2001]. Our result of elevated nuclear expression as a favorable prognostic factor in NSCLC seems inconsistent with their report. However, it must be kept in mind that Ape/Ref-1 is a multifunctional protein with a number of complex roles in maintenance of cellular health and genomic integrity [Flaherty et al., 2001]. It is involved in the repair of DNA damage, in the regulation of a variety of transcription factors [Tell et al., 2000], cell cycle control, hematopoiesis, and response to environmental stress. Therefore, there are still more questions to be answered about its regulation and role in cancer.

4.6 Other most often reported molecular tumor markers and prognosis of NSCLC

A large body of evidence indicates that multiple steps and multiple genes are involved in the progression of carcinogenesis, and multiple factors are involved in initiation and prognosis of cancer. Therefore, it should never be neglected to discuss the role of other important markers which have ever been previously testified to be related to prognosis of cancers.

4.6.1 p53 and NSCLC

Abnormal p53 gene products may be detected by immunohistochemistry in cancer cells as a consequence of p53 alterations. Our results demonstrated that 36.9% of NSCLC presented high level expression of p53. Moreover, p53 high level expression is more prevalent in squamous cell carcinoma than in adenocarcinoma of the lung and related to poor tumor cell differentiation. These findings are in accordance with most previous studies [Mitsudomi et al., 2000] and imply that the alteration of p53 gene causes a loss of tumor-suppressor function, promotes cellular proliferation and plays a major role in development and progression of NSCLC.

In a number of studies expression of oncoprotein p53 has been reported as a prognostic predictor in NSCLC with conflicting results. Some studies revealed a favorable prognosis in patients with low level expression of p53 protein [D'Amico et al., 2000; Moldvay et al., 2000; Nishio et al., 1996; Mitsudomi et al.,1993; D'Amico et al., 1999]. However, others [Apolinario et al., 1997; Cagini et al., 2000; Pastorino et al., 1997] did not demonstrate that positive p53 immunostaining is a predictor for poor prognosis in patients with NSCLC. The different immunohistochemical results between studies may be due to the procedures used, monoclonal antibodies, interpretation of expression levels, etc. Our univariate survival anlysis revealed that expression of p53 had significant prognostic value in adenocarcinoma of the lung, but not in squamous cell carcinoma and other types. This may be relevant to the following observations. Kawasaki found that nuclear p53 over-expression occurs at the transition from the early to advanced stage of replacement type adenocarcinoma development [Kawasaki et al., 1996]. In contrast, p53 overexpression is present in dysplasias, preneoplastic lesions of squamous cell carcinoma [Sozzi et al., 1992; Sundaresan et al., 1992]. This suggests that p53 alterations perhaps have different roles in adenocarcinoma and in squamous cell carcinoma, for example, p53 alteration is required for squamous carcinogenesis, whereas it plays a significant role in malignant progression of adenocarcinoma.

4.6.2 Ki67 and NSCLC

Ki67 is a nuclear antigen expressed during all periods of cell cycle but not in non-proliferating cells [Brown et al., 2002]. In this study, we found that 42.2% NSCLC tumors presented high level of Ki67 expression and the frequency of Ki67 high level expression was significantly higher in squamous cell carcinoma than that in adenocarcinoma of the lung. Additionally, significant association was found between Ki67 expression and the clinical stage, low level Ki67 expression was more frequent in stage than that in other stages. Consistent with these findings, there have been several studies on Ki-67 in NSCLCs. Viberti reported that in both biopsy and surgical specimens, high level expression of Ki-67 was associated with squamous cell carcinoma and poorly differentiated tumors [Viberti et al., 1997]. Moreover, Mehdi and colleagues investigated 260 patients with stage I and II NSCLCs and reported that Ki-67 expression was higher in squamous cell cancers than in nonsquamous cell cancers [Mehdi et al., 1998]. These findings, including our results, suggest that uncontrolled cell proliferation is the hallmark of malignant tumours, and the rate of cell proliferation is important in the development and progression of NSCLC.

Tumor proliferative activity has some relationship to an altered cell cycle. G_1 progression normally relies on stimulation by mitogenesis, and can thus be blocked by antiproliferative cytokines. On the other hand, abnormalities of the p53 protein may well be necessary for tumor development, and the p53 gene is reported to be affected by exposure to carcinogens [Sherr, 1996]. Therefore, we investigated whether there was a relationship between Ki67 expression and alterations of p53 protein that could regulate the proliferative activity of lung carcinomas. We found that there was a significant relationship between accumulation of p53 and high level expression of Ki67. This result, in agreement with a previous study [Haga et al., 2003], suggests that alterations of the p53 protein probably plays an important role in the proliferative activity of tumor cells, p53 overexpression might promote cell proliferation in NSCLC tumor cells. .

In confirmation of a previous study by D'Amico and coworkers [D'Amico et al., 2000], there was no significant relationship between the expression of Ki67 and overall survival of NSCLC. By contrast, Haga and coworkers evaluated Ki-67 expression specifically with respect to smoking status and found Ki67 expression and smoking status before surgery were important prognostic determinants in pulmonary adenocarcinoma [Haga et al., 2003]. We suggest that their results differ from ours because they just evaluated Ki-67 expression specifically with respect to smoking status and early stage NSCLC. Therefore, the prognostic role of Ki67 protein expression will require further investigated.

4.6.3 HER2 and NSCLC

Overexpression of the HER-2 proto-oncogene which encodes tyrosine kinase receptor p185neu has been observed frequently in many human cancers, including non-small cell lung cancer (NSCLC) [Meert et al., 2003]. Consistent with a most recent report [Nakamura et al., 2003], we found that 33.4% of NSCLC expressed HER2 at high level; and the frequency of high level expression of HER2 was higher in adenocarcinoma (38.2%) than in squamous cell carcinoma (25.8%). The frequency of HER2 high level expression was significantly higher in poorly differentiated tumor cells than in well differentiated tumor cells ($p=0.035$). This finding, in agreement with Bakir's report [Bakir et al., 2002], confirms that HER2 is related to development and progression of cancer. Our result also showed that over-expression of HER2 was statistically related to high level expression of p53. This finding has been observed very recently in breast cancer [Amashita et al., 2004], and suggests that alterations of the p53 protein play an important role in signal transduction, proliferative activity and apoptosis of tumor cells in NSCLC. The exact interaction between HER2 and p53 remains unclear at the present time, and needs to be further investigated.

Overexpression of HER-2 has been shown to enhance the metastatic potential by increasing the multiple adhesion and invasion steps of the metastatic cascade in an

experimental model [Yu et al., 1994; Ochiai et al., 1994; Bernstein et al., 1994] and to correlate with poor survival of patients with breast, ovarian, lung, stomach, or oral cavity cancer [Hung et al., 1999]. In the present study, we could not find any relationship between HER2 expression and overall survival of patients suffering from NSCLC, when comparing the high level expression group with the low level expression group. However, consistent with Pfeiffer's report [Pfeiffer et al., 1996], subset analysis according to cell types demonstrated that high level expression of HER2 was closely associated with unfavorable survival of lung squamous cell carcinoma, but not with adenocarcinoma. This difference of survival between different cell types in relation to HER2 expression may reflect the heterogeneity of biological behavior of tumors.

HER2 expression may be linked to a chemo-resistant phenotype in human NSCLC. Tumors cell lines overexpressing the HER2 protein have been shown to be more resistant to cisplatin [Tsai et al., 1995]. The possibility that adjuvant chemotherapy might improve the survival in patients with NSCLC makes HER2 an attractive target for patients with NSCLC. Recently, a recombinant humanized antibody against the extracellular domain of HER2 (Trastuzumab) was approved for the treatment of solid tumors. Also, the United States Food and Drug Administration approval was granted on the basis of findings on the use of the agent in the treatment of a subset of patients with metastatic breast cancer overexpressing the HER2 [Scheurle D et al., 2000]. A report indicated that high level expression of HER2 was due to gene amplification in NSCLC, and shared the same mechanism functioning in breast cancer [Hirashima et al., 2001], Therefore, it's rational that NSCLC patients with HER2 overexpression might benefit from the treatment of Trastuzumab. At present, several clinical trials concerning the role of Trastuzumab in the treatment of NSCLC are ongoing in the U.S.A. [Zinner et al., 2004].

4.6.4 E-cadherin, and ß-catenin and NSCLC

There is increasing evidence that modulation of the E-cadherin-catenin cell-cell

adhesion complex is an important step in the initiation and progression of human cancers. [Wijnhoven et al., 2000]. Recently, several clinical studies have suggested that dedifferentiation, metastasis, and reduced survival in NSCLC are results of reduced expression of E-cadherin and catenins [Bremnes 2002a]. However, the number of tumors and markers examined, follow-up time, and correlation with clinical data have been limited in these studies. Here, we present a large scale clinical study that efficiently used a high throughput TMA to evaluate the prognostic role of the E-cadherin-catenin adhesion complex in patients with NSCLC.

Our results revealed that 30.8% of NSCLC exhibited low level expression of E-cadherin. Additionally, squamous carcinoma had more frequently low level expression of E-cadherin than adenocarcinoma. In agreement with many previous studies describing the association between cadherin-catenin expression and clinico-pathological features [Kase et al., 2000; Liu et al., 2001; Pirinen et al., 2001; Kimura et al., 2000], our results demonstrate that the level of E-cadherin expression significantly correlates with tumor status (T status), lymph node status (N status), clinical stage and tumor cell differentiation. These findings suggest that abnormality of E-cadherin expression plays an important role in lung cancer cell differentiation, lung cancer invasion and metastasis.

Since ß-catenin, independently, plays a critical role in the regulation of cadherin-mediated adhesion, E-cadherin immunoreactivity alone will not always imply the presence or absence of a functionally normal cadherin-catenin complex [Pignatelli et al., 1994]. Thus, to evaluate the impact of this complex on tumor invasion and metastasis, it is necessary to assess the tumor cell expression of ß-catenin in addition to that of E-cadherin. In agreement with most previous studies, we found that 24.5% of NSCLC showed low level expression of ß-catenin. In the majority of studies [Nawrocki et al., 1998; Herbst et al., 2000], no associations between reduced ß-catenin expression and unfavorable pathologic TNM status have been found. However, our data showed that ß-catenin expression was related to lymph node status (N status). This

inconsistency may be due to the smaller number of cases in earlier studies.

Our finding that low level expression of E-cadherin is a predictor of poor prognosis of NSCLC is consistent with previous report [Bremnes 2002b]. Univariate analysis revealed that E-cadherin had important impact on lung cancer survival regardless of the histological cell type. Furthermore, multivariate survival analysis revealed E-cadherin to be an independent prognostic factor, together with lymph node metastasis, clinical stage, and differentiation. In accordance with reports from England and Taiwan [Ramasami et al., 2000; Lee et al., 2002], our results did not propose ß-catenin as an independent prognostic factor for NSCLC survival, possibly because of the strong correlation between the level of E-cadherin and ß-catenin expression.

In conclusion, the present study supports a functional relationship between E-cadherin and ß-catenin. Moreover, the poor prognosis in patients with low level expression of these molecules is considered to be due to adherence junction disassembly and, consequently, nonadhesive invasive and metastatic cells. However, these findings need to be confirmed in future studies.

4.7 Prospect

Advanced lung cancer is considered incurable and even the survival of patients with operable NSCLC is poor [Devesa et al., 1995; Mountain, 1997]. The problem of distinguishing patients into groups with different outcomes is one of major obstacles for improving the effectiveness of treatment. A large number of studies have been carried out attempting to seek out the reliable parameters which could not only functionally reflect the nature of the tumor but also efficiently predict the prognosis and treatment response, and subsequently improve diagnostic and prognostic information for patient management [Michael et al., 2002].

Although many prognostic factors have been proposed, none of these factors has been

accepted for routine clinical use due to conflicting and controversial results. Therefore, it was supposed that more biological markers in addition to disease stage are necessary to coherently stratify the survival or prognosis of NSCLC patients. As consequence, many studies have focused on the prognostic role of multiple factors with different functions [Fujino et al., 1995; Dosaka-Akita et al., 1997; D'Amico et al., 1999; Miyamoto et al., 1991; Kwiatkowski et al 1998]. In our study, we evaluated two DNA repair factors, as well as five most often reported factors. We provided E-cadherin as an independent prognostic factor for NSCLC, and found that low level expression of Ape/Ref-1and E-cadherin had a significantly unfavorable impact on NSCLC patient survival. In addition, Rad51, HER2 and ß-catenin had a prognostic role in squamous carcinoma of lung in contrast to p53 which influences outcome of lung adenocarcinoma. In future, a prospective study needs to be conducted to clarify the prognostic role of these factors.

Molecular prognostic research and biological substaging offer an opportunity to individualize a chemotherapeutic regimen based on the molecular profile of the tumor, thus providing the potential for improved outcomes with less morbidity in patients with NSCLC [Lau et al., 2002]. Taking into account the association of DNA repair capability and tumor resistance to therapy, the next step will be to examine the capability of alteration of DNA repair to predict the optimal regimen for NSCLC. Our further studies should prospectively investigate whether patients with high level Rad51 expression or/and low level Ape/Ref-1 expression really have poorer prognoses than the rest of patients, and whether patients with abnormal DNA repair can be a target for experimental therapeutic approach. Perhaps the most promising area of research is the development of novel drugs whose mechanism of action targets the pathways of these markers.

5 Summary

Altered expressions of Rad51, Ape/Ref-1, p53, Ki67, as well as E-Cadherin and ß-catenin have been reported to be involved in various human malignancies. This study is aimed at investigating the prognostic significance of these factors in non-small-cell lung cancer (NSCLC)

NSCLC tissue microarrays (TMAs) were constructed containing specimens from 383 consecutive patients. All patients underwent thoracectomy at a single institute. TMA slides were immunohistochemically stained. The expression of these markers was analyzed with relation to the clinico-pathological and survival data of the patients.

The results revealed that altered expressions of Rad51, Ape/Ref-1, p53, Ki67, as well as E-Cadherin and ß-catenin existed in NSCLC tumors. Univariate survival analysis demonstrated that high level expression of nuclear Ape/Ref-1 was a favorable prognostic factor for NSCLC ($p=0.040$). In contrast, high level expression of Rad51 was associated with shorter survival time of patients with squamous cell carcinoma ($p<0.001$). Moreover, our results confirmed that HER2 and ß-catenin had a prognostic role in squamous cell carcinoma ($p=0.001$ and $p=0.032$, respectively), whereas high level expression of p53 was a poor prognostic factor for adnocarcinoma ($p=0.040$), E-Cadherin proved to be an independent prognostic factor, together with stage, lymph node status and tumor cell differentiation by multiple survival analysis.

Our results suggest that altered function of Rad51 and Ape/Ref-1, as well as p53, Ki67, E-Cadherin and ß-catenin might play a crucial role in the development and progression of NSCLC. Our findings provide factors which are helpful for identifying patients with high risk of recurrence or metastasis and might assist the clinician in decision making of an appropriate therapy for the individual patient. These markers are also considered good targets for the development of novel anticancer drugs.

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8 Own publications

papers on non-small cell lung cancer

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3. Qiao Guibin, Wu Yilong. Molecular targeting therapy of Lung cancer, *Chinese Lung Cancer*, 2002;(4):23
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