AICHI CANCER CENTER
INTERNATIONAL SYMPOSIUM IV

The Cutting Edge of Lung Cancer Research:
From Benchtop to Bedside

January 31, 1998
International Conference Hall
Aichi Cancer Center
Nagoya, Japan
PROGRAM AND ABSTRACTS

THE 4TH AICHI CANCER CENTER INTERNATIONAL SYMPOSIUM

THE CUTTING EDGE OF LUNG CANCER RESEARCH: FROM BENCHTOP TO BEDSIDE

Committee of the Aichi Cancer Center International Symposium

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January 31, 1998

Aichi Cancer Center
Nagoya
Japan
PROGRAM OF SYMPOSIUM

Opening Remarks
9:20-9:30 Makoto Ogawa (Aichi Cancer Center)

Keynote address: (Chairperson: Takashi Takahashi, Aichi Cancer Center)
9:30-10:20 Molecular pathogenesis of lung cancer
John D. Minna (Hamon Center for Therapeutic Oncology, Research)

Epidemiology: (Chairperson: Jin S. Lee, M.D. Anderson Cancer Center)
10:20-10:50 Epidemiological hints for the fight against lung cancer
Hiroyuki Shimizu (Gifu Univ.)

Molecular Pathogenesis 1: (Chairperson: Eiju Tsuchiya, Saitama Cancer Center)
10:50-12:00 Recent advances in the molecular cytogenetic analysis of human lung carcinomas
Joseph R. Testa (Fox Chase Cancer Center)

Heterogeneity of lung cancer in relation to morphological changes and molecular abnormalities
Masayuki Noguchi (Univ. of Tsukuba)

12:00-13:15 Lunch

Molecular Pathogenesis 2: (Chairperson: Joseph Testa, Fox Chase Cancer Center)
13:15-14:15 Molecular pathogenesis updates of lung cancer
Jun Yokota (National Cancer Center)

Transforming growth factor-β and the Smad genes in the pathogenesis of lung cancer
Hirotaka Osada (Aichi Cancer Center)
Prevention and Diagnosis:
(Chairperson: David P. Carbone, Vanderbilt Cancer Center)

14:15-15:40 Non-steroidal anti-inflammatory drugs in prevention and
treatment of lung cancer
Toyoaki Hida (Aichi Cancer Center)

New directions in lung cancer chemoprevention
Jin Soo Lee (M.D. Anderson Cancer Center)

p53 in molecular diagnosis of lung cancer
Tetsuya Mitsudomi (Aichi Cancer Center)

15:40-16:05 Coffee Break

Innovative Treatment: (Chairperson: Nagahiro Saijo, National Cancer Center)

16:05-17:50 Dendritic cells and host-tumor interactions in T-cell targeting of
human solid tumors
David P. Carbone (Vanderbilt Cancer Center)

Heavy ion therapy.: a magic bullet in lung cancer treatment?
Hirohiko Tsuji (National Institute of Radiological Sciences)

Gene therapy of lung cancer in Japan: an update and future
expectations
Toshiyoshi Fujiwara (Univ. of Okayama)

Concluding Remarks

17:50-17:55 Suketami Tominaga (Aichi Cancer Center)
Welcome Remarks

Makoto Ogawa

President, Aichi Cancer Center

On behalf of the organizing committee, I would like to welcome all of you to the fourth Aichi Cancer Center International Symposium. The first international symposium was held in 1994 when Aichi Cancer Center celebrated the 30th Anniversary. Since then, the symposium has been held annually. Topics discussed in the past symposiums were "From prevention to treatment", "Role of DNA transactions in carcinogenesis" and "Recent advances on hepatobiliary-pacreatic cancer". This year, the organizing committee selected a title of "The cutting edge of lung cancer research". Lung cancer is the most common and lethal malignancy in Japan and over 48,000 patients died of this malignancy in 1996. The majority of patients present with metastatic disease, and therefore the overall cure rate is low. This symposium starts from keynote address entitled "Molecular Pathogenesis of Lung Cancer", and subsequently, epidemiology, molecular pathogenesis, prevention and diagnosis, and treatment will be discussed in depth. I hope this symposium will stimulate the translational research on lung cancer.
MEMO
Molecular Pathogenesis of Lung Cancer

John D. Minna, M.D., Yoshitaka Sekido, M.D., Ph.D., Kwun M. Fong, M.D., Monja Proctor, M.D., Eric Biesterveld, Zhao Wu, Ph.D., Anu Bansal, Ph.D., Joseph Geradts, M.D., Eva Forgacs, Gina M. Melc, Arvind K. Virmani, Ph.D., Ivan I. Wistuba, M.D., Adi F. Gazelar, M.D.

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Studies from many laboratories identifying molecular genetic changes in lung cancers and their associated preneoplastic respiratory epithelial lesions have led to the following conclusions and suggestions for potential transnational research applications:

Lung cancer arises because of mutation in dominant and recessive oncogene. This has been documented by molecular genetic studies demonstrating abnormalities of these genes or the expression of their products in invasive and metastatic cancers. There are many genetic changes in each lung cancer. A conservative estimate is that a clinically obvious primary lung cancer can have 10-20 such changes. If preneoplastic lesions with a few but not all changes can be identified this may provide very early molecular diagnosis and allow for very early treatment. Mutations in dominant and recessive oncogenes can occur by many mechanisms including point mutation, splicing error, deletion, rearrangement, gene amplification and deregulated expression (over expression of dominant oncogenes and lack of expression of recessive oncogenes) by as yet unknown mechanisms. A recently discovered mechanism involving inactivation of recessive oncogene expression is hypermethylation best studied in inactivation of the p16 recessive oncogene. If silenced recessive oncogene expression can be reactivated pharmacologically as has been done with other examples of methylation repression this could represent a novel form of therapy. For those mutations altering the amino acid sequence of the proteins absolute differences are created between tumor and normal tissues. These can be exploited as targets for development of new drugs, and also for developing
cancer specific vaccine therapies which are currently being tested in lung cancer patients using the patient's rumor own mutant p53 and peptides pulsed onto the patient's dendritic cells.

**Identification of 3p recessive oncogenes in lung cancer.** Chromosome region 3p is the most frequent site of allele loss in lung cancer. In addition, 3p allele loss appears to be the earliest change found even in normal appearing epithelium. There are several different 3p recessive oncogenes involved in the pathogenesis of lung cancer located at: 3p12-13(U2020 homozygous deletion);3p 14.2 FHIT homozygous deletion region and the FRA3B fragile site; 3p21 (BAP-1, BRCA1 binding protein); 3p21.3 (H740/H1450)/GLC20 homozygous deletion);3p21.3 (ACL5 homozygous deletion region); and the 3p25 VHL gene region. In invasive lung cancer the 3p allele loss usually uncovers all of these 3p recessive oncogene loci (from 3p13 to the telomere), while in preneoplastic lesions, there appears to be more localized 3p loss of heterozygosity. Thus, it is likely that the invasive tumor requires the inactivation of several 3p recessive oncogenes. **Identification of multiple new sites of recessive oncogenes.** Genome wide allelotyping as well as the discovery of new candidate genes has greatly, increased the number of new recessive oncogenes with abnormalities in lung cancer distributed on several different chromosomes.

When DNA sequence changes in the same gene can be studied in many different tumors, (such as in the p53 or ras genes) the type of mutations are usually consistent with causation by carcinogens in cigarette smoke. Typical mutations associated smoke carcinogens are G to T transversions found in p53 and ras mutations. In addition, the exact pattern of mutations in p53 found in lung cancers are induced by benzopyrenes in p53 genes in normal tissues. These mutation profiles provide an absolute connection between cigarette smoke carcinogens and lung cancer. This information will be very important in dealing with tabacco companies.

**All of the genetic changes in dominant and recessive oncogenes appear to be required for maintenance of the malignant phenotype.** In selected cases, it has been possible to "correct" one of these defects (such as introduction of a wild-
type p53 gene into a lung cancer cell line with a p53 mutation) and cause the cells to become non-tumorigenic despite the presence of multiple other genetic defects. Even better, gene therapy trails in patients by local injection of viral vectors carrying wild-type p53 have shown tumor regressions in humans providing proof of principle.

**Lung cancers are characterized by genetic instability suggesting novel lesion in DNA repair and/or synthesis.** Besides the large number of chromosomal changes, there is recent information that shows genomic (also called microsatellite) alterations involving DNA repeat sequences to occur relatively, frequently in lung cancer and preneoplastic lesion DNA. This appears to be related but distinct from the microsatellite instability seen in the replication error repair phenotype characteristic of mutations in one of the DNA mismatch repair enzyme genes. Lung cancer cells also have abnormalities in the base excision repair (BER) system which account for some of the mutations. It will be important to understand the nature of the lesions in DNA repair and/or synthesis leading to such instability and genomic alternations. In the meantime, the presence of these microsatellite alterations are being used for molecular detection of cancer and preneoplastic lesions.

**Lung cancers express the enzyme telomerase providing a mechanism for cellular immortality.**
There is nearly universal expression (>90%) of the enzyme telomerase and the RNA component of telomerase in primary tumor samples and carcinoma in situ. Shortening of telomers occurring during normal cell DNA replication leads to cellular "mortality" while expression of telomerase stabilizes telomers and its associated with unlimited cell growth ("Immortality"). Telomerase and telomerase RNA can be used for early detection studies and represent an attractive target for developing new therapies.

**Apoptosis (programmed cell death) is abnormal in lung cancer cells.** Lung cancer cells express products such as Bcl-2 which can inhibit apoptosis (programmed cell death). More studies are needed on recently discovered components of the apoptotic pathway as well as in determining the time in lung
cancer pathogenesis when changes in apoptosis occur. In this regard, there is evidence that nicotine is able to antagonize apoptotic signals in lung cancer and other cells by acting through nicotinic acetylcholine receptors. Thus, it is possible that nicotine ingested through cigarette smoking may play a role in blocking signals for programmed cell death during lung cancer pathogenesis. It is also possible that carcinogenic derivatives of nicotine may also interact with these receptors in a manner to facilitate carcinogenesis. Lung cancer cells also express receptors for a variety of opioids, and opioids, acting through these receptors induced apoptosis in lung cancer cells (which nicotine reverses).

Cell regulation pathways disrupted in lung cancer cells may have mutations in several different components if the pathway. Mutations in dominant and recessive oncogene products disrupt several cell regulation pathways and mutations often occur in different components of the same pathway. Pathways which appear frequently involved in lung cancer are the MAP kinase pathway which has as some of its components the products of the Her2/neu, ras and myc dominant oncogenes and the p53 recessive oncogene all of which can be mutated in lung cancer; the p53 pathway involved in response to DNA damage and including the ATM (ataxia telangectasia mutated), and p21/WAF1/CIP1 gene products. and the retinoblastoma RB/p16(CDKN2) pathway governing the G1/S checkpoint boundary. It will be important to know if we can take advantage therapeutically of these deranged pathways to develop new therapeutics (targeted against the mutated product), or strategies which make use of these abnormalities to help activate apoptosis in the cancer cells.

Molecular Changes are Frequently Found in Preneoplastic Lung Lesions. A series of morphologically distinct preneoplastic changes (hyperplasia, dysplasia, and carcinoma in situ) can be observed in respiratory epithelium of current and former smokers and of lung cancer patients. Allelotyping of precisely microdissected preneoplastic foci of cells shows that 3p allele loss is the earliest change detected followed by 9p allele loss (p16/CDKN2 gene), 17p (p53 gene) allele loss, and ras mutations. It is striking that both current and former smokers have easily detectable clones demonstrating allele loss in ≥50% of individuals while never smokers don't have these changes. This is striking evidence of a
field carcinogenesis effect and will allow for monitoring of changes in chemoprevention trial, and may also allow for molecular early detection of persons at greatest risk of developing lung cancer. The genetic relatedness of these lesions is also apparent in the phenomenon of allele specific loss. This either represents a clone of cells spreading throughout the lung, or some tissue specific occurrence such as imprinting to give genetically similar populations of otherwise dispersed cells.

*Retinoid resistance is frequent in lung cancers.* Lung cancers are frequently resistant to retinoids and there are several mechanisms underlying this resistance. One striking example is loss of expression of RAMPβ receptors in lung cancers. It will be important to learn if exogenous treatment with retinoids can reactivate this expression and whether loss of expression occurs in preneoplastic lesions. Nevertheless, this resistance strongly suggests that normally endogenous retinoids function to prevent lung cancer and that clinically evident lung cancer represents an "escape" from this effect.

*Lung cancers produce paracrine factors which aid in their pathologic behavior.* In addition, to autocrine growth loops present in lung cancer and discussed elsewhere in this symposium, lung cancers produce vascular endothelial growth factor (VEGF) and FAS ligand. VEGF works to both stimulate tumor angiogenesis and to block maturation of dendritic giving an immune defect. FAS ligand similarly paralyzes the immune system by peripheral deletion of tumor-reactive T-cell clones.

**Acknowledgements:** We gratefully acknowledge the many current and past collaborators and past postdoctoral fellows who have worked in our laboratory studying lung cancer. We make special note of the important discoveries of Takahashi Takashi, M.D. and Tetsuya Mitsudomi, M.D. of the Aichi Cancer Center Research Institute as well as the other Japanese Postdoctoral Fellows who worked in our labs.
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Epidemiological Hints for the Fight Against Lung Cancer

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Exclusion of risk factors of lung cancer from our environment must be directly, associated with prevention of the disease. However, the evidence as etiology for each factor is not always strong and difficulty for excluding the factor varies up to the characteristics of the factor itself and cultural, political and economical conditions.

After the outlook on the descriptive epidemiology of lung cancer we will review scientific articles on the association between lung cancer and some classical risk factors such as cigarette smoking and asbestos, and try to calculate the attributable risk percent discussing the validity of the data. We will also show the factors like beta-carotene that can protect or inhibit of developing lung cancer and evaluate them.

Recent development of molecular epidemiology is apparent in the field of lung cancer and polymorphism of p450 as an etiologic factor has been discussed frequently. The Japanese studies provided evidence that genetic susceptibility ascribable to CYP1A1 and GSTM1 depends on dose level of cigarette smoking. There is an expanded possibility of preventive control when an interaction occurs between smoking and these factors. However, these studies including few non-smokers in cases make it difficult to estimate relative interaction magnitude precisely. We cannot evaluate these genetic factors at the same level as classical etiologic factors like smoking at this moment.

The other approach for the fight against lung cancer before active treatment is early, detection of lung cancer. One of the famous randomized controlled trials, Mayo Lung Project, did not show the positive effect of screening for decreasing deaths from lung cancer. However, the results from a case-control study in Japan indicated the effectiveness of the screening program. We will compare the usefulness of the mass screening and smoking cessation program from several points of view.
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Recent Advances in the Molecular Cytogenetic Analysis of Human Lung Carcinomas

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Karyotypic studies have revealed multiple cytogenetic changes in most small cell lung carcinomas (SCLCs) and non-small cell lung carcinomas (NSCLCs). In SCLCs, losses from 3p, 5q, 13q, and 17p predominate; double minutes associated with amplification of members of the MYC oncogene family may occur late in disease. In NSCLCs, deletion of 3p, 9q, and 17p, +7, i(5p), and i(8q) are often reported. The recurrent deletions encompass sites of tumor suppressor genes commonly inactivated in lung carcinomas, e.g., CDKN2 (9p21), RB1 (13q14), and TP53 (17p13). Despite technical advances in cell culture, the rate of successful karyotypic analysis of lung carcinomas has remained low. However, recent molecular cytogenetic methods hold promise for significant improvements in the assessment of chromosome changes in lung cancer. Interphase fluorescence in situ hybridization (FISH), using centromeric DNA probes specific for individual chromosomes, is a rapid procedure for detecting numerical chromosome changes in lung tumors and can be particularly useful in the analysis of small or non-sterile specimens. In NSCLC, such studies have revealed a number of recurrent changes such as gain of chromosome 7. Another technique, comparative genomic hybridization (CGH), has proven valuable for assessing chromosomal imbalances within entire tumor genomes. This technique does not require mitotic tumor cells and can be used to detect chromosomal gains or losses and identify the location of amplified genes. CGH accounts for all chromosomal segments, including those present in marker chromosomes whose origin cannot be determined by karyotypic analysis. In SCLC, CGH analysis has identified several new recurrent abnormalities, such as loss from 10q and overrepresentation of 3q, and several recurrent sites of DNA amplification. Our CGH analyses of NSCLCs have documented frequent losses from 3p, 5q, 8p, 9p, 17p, and 18q, in agreement with prior karyotypic and LOH studies. In addition, we identified several sites of copy number increases whose
high frequency had not been previously recognized. Prominent among these was overrepresentation of 3q; extra copies of part or all of 5p, 7p and 8q were also very common, and in many instances these overrepresented regions were present at high copy numbers. Other investigators have reported distinct CGH patterns in lung adenocarcinomas and squamous cell carcinomas. Prominent differences include overrepresentation of 1q and deletion of 9q22 in adenocarcinomas and loss of 2q36-37 and overrepresentation of 3q in squamous cell carcinomas. Two additional technical innovations, multiplex-FISH (M-FISH) and multicolor spectral karyotyping (SKY), use a pool of painting probes, each labeled with a different fluor combination, to specifically identify each human chromosome. M-FISH and SKY permit the identification of cryptic translocations and the characterization of complex structural rearrangements whose origin cannot be determined by conventional karyotyping. The further application of such molecular cytogenetic approaches in combination with other recent advances, e.g., laser imaging fluorescence endoscopy and laser capture microdissection, opens new avenues for the analysis of early bronchial lesions and selected tumor cell populations. Such studies will facilitate a better understanding of the role of chromosome alterations in the pathogenesis of lung cancer.
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Heterogeneity of Lung Cancer in Relation to Morphological Changes and Molecular Abnormalities

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Loss of heterozygosity, (LOH) was examined using polymerase chain reaction (PCR) amplification of microsatellite loci in relatively early stage adenocarcinoma classified according to recently proposed histological criteria (Noguchi M et al. Cancer 1995; 75: 2844-2852) which divided adenocarcinomas into six subtypes (A; localized bronchioloalveolar carcinoma (LBAC), B, LBAC with alveolar collapse, C; LBAC with active fibroblastic proliferation, D; poorly differentiated adenocarcinoma, E; tubular adenocarcinoma, F. true papillary adenocarcinoma).

The frequencies of LOH were 19.8% in types A and B, 26.8% in type C, and 32.7% in type D tumors. There were no significant differences in the frequency of LOH on chromosome 2p, 3p, 9p, 17q among tumor types. However, on 17p, the frequency of LOH was significantly lower for types A and B than for type C or D. Six type C adenocarcinomas were examined and three exhibited different LOH patterns at central region showing invasive growth and peripheral region showing replacement growth. These results indicate that heterogeneous genetic alterations can be demonstrated even in an early stage adenocarcinoma.

Allelic imbalances of lung carcinoma examined by arbitrarily primed PCR and background abnormalities of the pulmonary adenocarcinomas will also be discussed.
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<th>Year</th>
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Molecular Pathogenesis Updates of Lung Cancer

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Recent advances in molecular genetics of human lung cancer have revealed that several tumor suppressor genes (TSGs) are involved in multistage lung carcinogenesis. In particular, inactivation of the p53 gene as well as loss of heterozygosity (LOH) on chromosome 3p occurs commonly in all histological types of lung cancers. RB inactivation is frequent in small cell lung carcinoma (SCLC), while the p16 gene is preferentially inactivated in non-small cell lung carcinoma (NSCLC). However, no other TSGs have been identified as being involved in lung carcinogenesis, and there is little knowledge about the sequence of genetic events that accumulate during lung cancer progression. In addition, pathogenetic significance of each genetic alteration in lung carcinogenesis remains unclassified.

To obtain information about the sequential TSG inactivation that accumulates in the course of lung cancer progression, we have screened for LOH on all autosomal chromosomes in various stages of lung cancers. The incidence of LOH on chromosomes 3p, 13q and 17p was high in any progression stages of NSCLC as in the case of SCLC. In SCLC, LOH also occurs frequently on chromosomes 5q and 22q irrespective of the stages. In advanced NSCLC, a high incidence of LOH was observed at loci on chromosomes 2q, 9p, 18q and 22q, and the incidence of LOH on these chromosomes in brain metastases was significantly higher than that in stage I primary tumors. Thus, it was indicated that TSGs on chromosomes 2q, 9p, 18q and 22q play an important role in the acquisition of more malignant phenotypes in NSCLC, while TSGs on chromosomes 5q and 22q may function for the aggressiveness of SCLC.

Since a number of tumor suppressor genes have been isolated from chromosomal region showing homozygous deletions in cancer cells, we have been searching for loci showing homozygous chromosomal deletions in lung cancer cells by several molecular genetic methods. Up to present, homozygous
deletions have been detected at several chromosomal loci, including chromosomes 2q, 9p and 21q, in lung cancer cells. We are currently searching for genes mapped to the region of the homozygous deletions and inactivated in lung cancer cells. Results of molecular analysis on the homozygously deleted regions will be presented. On the basis of the results obtained in our laboratory, I will discuss the pathogenetic significance of TSG inactivation in lung carcinoma progression and the application of molecular genetic information for diagnosis and treatment of lung cancer.

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Transforming Growth Factor-β and the Smad Genes in Pathogenesis of Lung Cancer

Hirotaka Osada

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Transforming growth factor-β (TGF-β) family regulates a remarkable range of biological activity including cell proliferation and differentiation, tissue morphogenesis and apoptosis. TGF-β signals through membrane serine/threonine kinase receptors, which phosphorylates the intracellular components to propagate the signal. Recently as intracellular components of TGF-β family signaling pathway, Smad gene products have been identified. Smad genes are now known to make a family consisting of 7 members, among which Smad2, Smad3, and Smad4 proved to be mediators of TGF-β responses including suppression of cell proliferation. Alteration of TGF-β signaling pathway is also considered to be involved in tumorigenesis, because human cancer cell lines have been reported to lose the responsiveness to TGF-β growth inhibitory effect as tumor aggressiveness increases. Human immortalized lung epithelial cell line BEAS-2B (a generous gift from C. Harris) and HPL1, established in our laboratory by A. Masuda et al, showed marked responses to TGF-β, including cell growth inhibition and induction of fibronectin expression and actin stress fiber formation. In contrast, in our preliminary study, loss of responsiveness to TGF-β seemed to be frequent in a major fraction of human lung cancer cell lines. Therefore, it is suggested that acquisition of TGF-B resistance may play an important role in lung cancer development.

Human SMAD2 and SMAD4/DPC4 genes are closely, located at 18q21 region. Within this 18q21 region, tumor suppressor gene candidates, DCC and maspin, and bcl-2 oncogene have been already mapped, although the involvement of these genes in lung tumorigenesis have not been demonstrated clearly so far. Frequent allelic losses of this region in lung cancers also previously reported by J. Yokota's group. To investigate further whether a tumor suppressor gene for lung cancer resides at 18q21 locus, first investigated allelic
loss and methylation status at 18q21 in 134 lung cancer specimens using bcl-2 gene probe. Allelic loss at the bcl-2 locus was observed in 25% (12/49 informative cases) of non-small cell lung cancers, and most frequently detected in adenocarcinomas (40%, 7/17 informative). Aberrant hypermethylation was also seen in 23% (28 out of 120 cases) of non-small cell lung cancers. These results suggested the existence of a putative tumor suppressor gene for lung cancer at 18q21.

To investigate whether SMAD2 and SMAD4/DPC4 is a tumor suppressor gene involved in lung cancer development, we then examined 57 lung cancer specimens taken directly from patients for status of SMAD2 and SMAD4/DPC4 genes. The study of SMAD4/DPC4 gene demonstrated two missense somatic mutations in the C-terminal conserved region, MH2 and a 2-bp frameshift mutation between the N-terminal conserved region, MH1, and MH2 region. In SMAD2 gene analysis, a missense somatic mutation and a 9-bp in-frame deletion in the MH2 region were detected. These findings strongly suggested that SMAD4/DPC4 and SMAD2 may function as tumor suppressor genes at 18q21 locus in lung cancers. The incidence (9%) of alterations in DPC4/SMAD4 and SMAD2 genes is, however, not sufficient to account for the frequent deletion at 18q21 locus in lung cancers, suggesting that another tumor suppressor gene may exist in this chromosome region.

To examine functional inactivation of these in vivo mutations of SMAD4/DPC4 and SMAD2, we cloned cDNAs of SMAD4/DPC4 and SMAD2 genes from lung cancers in which in vivo mutations were detected, and made the constructs expressing each mutant SMAD4/DPC4 or SMAD2 gene. The function of the mutants to mediate TGF-β signaling was studied using PAI-1 reporter assay. All SMAD4/DPC4 and SMAD2 mutants identified in lung cancers demonstrated inability to mediate transcriptional activation of PAI-1 promoter by TGF-β signal, suggesting that these mutations indeed disrupted TGF-β signaling and may have contributed to the pathogenesis of the lung cancer cases.

In contrast to SMAD4/DPC4 and SMAD2 gene alteration, we found that mutation in SMAD3 gene was extremely rare in lung cancers if at all present. Further investigations appear to be necessary to clarify the whole mechanism of frequent TGF-β unresponsiveness in lung cancers. The complete
understanding of the molecular events in loss of TGF-β responsiveness in lung cancers could provide an insight into the mechanism of lung cancer development.

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Non-steroidal Anti-Inflammatory Drugs in Prevention and Treatment of Lung Cancer

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Non-steroidal antiinflammatory drugs (NSAIDs) are potent inhibitors of COX enzymes that catalyse the synthesis of prostaglandins from arachidonic acid. Several lines of evidence now support the notion that NSAIDs may prevent human cancer. We previously showed that NSAIDs, such as aspirin or indomethacin, reduced PGE$_2$ levels and the growth of non-small cell lung cancer (NSCLC) cells both \textit{in vivo} and \textit{ex vivo} in nude mice. Furthermore, lung adenomas induced by urethane in A/J mice were shown to be partially inhibited by the administration of indomethacin. These results suggested that the cyclooxygenases (COX) pathway may be involved in the development of lung cancer.

Two types of COX enzymes, COX-1 and COX-2, have been identified thus far, while a possible link between upregulated expression of COX-2 and colon carcinogenesis has been suggested in several studies. We examined expression of COX-2 in human lung cancer cell lines as well as in tumor specimens taken directly from patients. The expression of COX-2 were readily detectable in 6 of 10 NSCLC cell lines and in 1 of 16 small cell lung cancer (SCLC) lines by Northern blot analysis, whereas it was not detected in both normal bronchial (BEAS2B) and peripheral lung (HPL1D) epithelial cell lines even by more sensitive RT-PCR analysis. Our preliminary immunohistological analysis using a COX-2-specific antibody also showed significant positive staining in cancer cells.

In conclusion, these data suggest that the COX enzymes may, be important regulatory, components of NSCLC, and COX-2 inhibitors might be useful as a possible chemopreventive agent in NSCLC, especially in adenocarcinoma. The intervention with COX-2 inhibitor may prevent lung carcinogenesis, and the development of effective chemoprevention may allow
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New Directions in Lung Cancer Chemoprevention

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Lung cancer is an increasingly important public health problem throughout the world. Despite continuing efforts to promote smoking cessation, the risk of lung cancer remains high and this risk continues for more than a decade after successful smoking cessation. Once cancer develops, the overall treatment outcome remains disappointingly poor, as reflected by a five-year survival rate of 13%. In addition, earlier hopes to reduce lung cancer mortality by detecting the lung cancer in earlier stages with regular chest radiographs and/or sputum cytology have not been fulfilled. Consequently, as a means to reduce the lung cancer mortality, renewed interest has been directed to chemoprevention. Chemoprevention is based on three basic premises: field cancerization, multi-step carcinogenesis, and availability of effective chemoprevention agents. The ultimate goal of chemoprevention is to reduce the risk of cancer development in the field of carcinogen exposure. One of the most touted and most extensively evaluated non-toxic natural products is beta-carotene, which has been the major component included in several chemoprevention trials. To date, two large randomized primary prevention trials faded to show any beneficial effect of beta-carotene. Instead, the results suggested that beta-carotene might do more harm than good in active smokers.

Obviously, a placebo-controlled intervention trial is needed to demonstrate the true efficacy of chemoprevention. Nevertheless, because the study end point (i.e., lung cancer) is an infrequent event even in such high-risk groups as heavy smokers, such trials require a large number of healthy volunteers and many years of follow-up. To overcome some of the inherent problems associated with trials that use cancer incidence as a study end point, there has been a surge of interest in defining biomarkers which can be used as intermediate end points. The basic premise is that, if a specific biomarker is known to be associated with carcinogenic process and its expression is frequently noted in the tissue at risk, its reversal would be linked eventually to a
reduced risk of cancer development. Taken together with the findings that almost one-half of all lung cancers are diagnosed in former smokers, at least in the United States, new directions are now directed toward biomarker-oriented short-term intervention trials in former smokers, using a bronchial biopsy-based chemoprevention trial as a model system.

In addition, patients who are cured of the first smoking-related primary cancer have been shown to have a much higher risk of developing another cancer (i.e., second primary, tumors: SPT), than even the active smokers do. These patients are the ideal candidates for chemoprevention trials. At least two lung cancer SPT chemoprevention trials have completed accrual and are waiting for data maturing and analysis. A pivotal head and neck SPT chemoprevention trial that was designed to evaluate the efficacy of 13-cis-retinoic acid has enrolled more than 86% of the accrual target of 1302 head and neck cancer patients. All the data available from our short-term biomarker-oriented chemoprevention trials and other investigators have provided indisputable support for the concept of field cancerization and multi-step carcinogenesis. In addition, there exists data strongly suggesting that cigarette smoke has different effects on different genes. Further expansion of these biomarker-oriented chemoprevention trials and exploration of additional panels of biomarkers will generate more exciting results in the field of lung cancer prevention. Moreover, expect that the findings obtained from the SPT trials will be directly applicable to the design of future primary chemoprevention trials.
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**p53 in Molecular Diagnosis of Lung Cancer**

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Alteration of the p53 tumor suppressor is one of the most frequently seen molecular abnormalities in human lung cancer. In our laboratory, efforts have been made to detect multifaceted p53 alterations in various clinical materials and to translate the findings into clinics.

1) **p53 analysis shows close relationship between smoking and lung cancer:** We analyzed lung cancer in 70 non-smoking patients for p53 mutations. Mutations were present in 18 (26%). In contrast to frequent G:C to T:A transversion in smoking patients, there was no such preferential pattern in non-smoking patients. This fact further substantiates importance of smoking cessation for prevention of lung cancer.

2) **p53 molecular diagnosis reveals clonal origin of multiple lung cancer:** We tried to distinguish multiple primary lung cancer from recurrent lung cancer by utilizing p53 mutation as a clonal marker. Sixteen patients who underwent multiple pulmonary resections for a suspected recurrent lung tumor or a multiple primary tumor were examined. Nine of the 16 cases had at least one p53 mutation in their tumors. The mutational status of the p53 gene was discordant in all nine patients, suggesting a different clonal origin despite the fact that six of them had almost identical histologic features. Analysis of p53 gene mutations was thus useful in diagnosis of multiple primary tumors.

3) **Auto-antibodies against p53 protein in sera of NSCLC patients:** We examined 188 consecutive patients with NSCLC for auto-antibodies against p53 by enzyme-linked immunosorbent assay. p53 antibodies were detected in 34 patients of 188 (18%). Patients with squamous cell carcinoma or those with
stage III-IV disease had a significantly higher incidence of \( p_{53} \) antibodies. Prevalence of \( p_{53} \) antibodies in patients with tumors having abnormal \( p_{53} \) protein accumulation was higher than that in patients without \( p_{53} \) abnormality (28% vs 14%). However, our data did not indicate clinical usefulness of \( p_{53} \) antibodies as a marker for relapse or prognosis of NSCLC.

4) \( p_{53} \) alteration as a prognostic factor of NSCLC: We evaluated a prognostic impact of \( p_{53} \) overexpression in 208 surgically treated NSCLC patients. Abnormal accumulation of \( p_{53} \) was a significant poor prognostic factor in a cohort of adenocarcinoma, while it was not predictive for poor prognosis in overall patients or in a cohort of squamous cell carcinoma. It is necessary to examine clinical usefulness of detection of \( p_{53} \) abnormality in predicting patients' outcome and in clinical decision making regarding postoperative adjuvant therapy in a prospective clinical protocol.

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Dendritic Cells and Host-tumor Interactions in T-cell Targeting of Human Solid Tumors

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Somatically acquired mutations in genes which directly, regulate tumor cell growth, such as p53, ras, and Rb, are well characterized in human solid tumors, but the tumor cell's acquisition of characteristics which alter its interface with the host have been less well studied.

We have reported that a major reason for the lack of host responses to tumor antigens is a tumor-associated defect in the host "professional antigen presenting cell" or dendritic cells\textsuperscript{1,2}. Further, this effect can be reproduced in vitro by differentiating hematopoietic precursor cells (HPC) to dendritic cells in the presence of tumor cell supernatents (TCS). We have discovered that vascular endothelial growth factor (VEGF), a molecule produced by most tumors and responsible for the induction of tumor neovasculature, also appears to be one of the most important factors in TCS with a selective inhibitory, effect on the ability of bone marrow precursors to differentiate into functional dendritic cells\textsuperscript{3}. This knowledge suggests that the combination of anti-angiogenic blockade of VEGF and immunotherapy, could be synergistic, and preliminary, results in animal model systems suggest that this is the case.

We have begun to investigate the mechanisms involved in the inhibition of DC differentiation by VEGF and TCS\textsuperscript{4}. Recently, we have demonstrated specific binding of VEGF to HPC. This binding was efficiently competed by Placenta Growth Factor (PlGF), a ligand reportedly specific for the F1t-1 receptor. The number of binding sites for VEGF decreases during DC maturation \textit{in vitro} associated with decreased levels of mRNA for F1t-1. VEGF significantly, inhibits NF-κB-dependent activation of reporter gene transcription during the first 24 hours in culture. The presence of VEGF significantly
decreased specific DNA binding by NF-κB as early as 30 min after induction with TNFα. This was followed on day 7-10 by decreases in the mRNA for RelB and cRel, two subunits of NF-κB. Blockade of NF-κB activity in HPC at early stages of differentiation with an adenovirus expressing a dominant IκB inhibitor of NF-κB reproduced the pattern of effects observed with VEGF. Thus, NF-κB appears to play an important role in maturation of HPCs to DC, and VEGF activation of the Flt-1 receptor is able to inhibit the activation of NF-κB in this system. Blockade of NF-κB activation in HPCs by tumor-derived factors may therefore be a mechanism by which tumor cells can directly down-modulate the ability of the immune system to generate effective antitumor immune responses.

To begin to test the therapeutic impact of p53 and ras targeted cellular immunotherapy, we have conducted a clinical trial of peptide immunization in patients with advanced cancer. To date, 30 patients have been treated, and this approach has been shown to be safe with an immunological response rate of 40%, quite good in this population of advanced cancer patients. Recent findings from the laboratory and the next generation of immunotherapeutic trials in lung cancer will be discussed.

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Heavy-Ion Therapy: A Magic Bullet in Lung Cancer Treatment?

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In radiotherapy of lung cancer, preservation of respiratory functional is of paramount importance, which has been achieved with improved dose distribution using conformal radiotherapy or modern 3-D irradiation techniques. In this regard, heavy-ions (carbon, neon ions) have the beneficial property of superior dose localization due to exhibiting a Bragg peak curve in the body, and of greater biological effectiveness than low-LET radiations (proton, photon). Accordingly, it is expected that heavy-ions would be effective against locally advanced, radioresistant tumors and those located near critical structures. The initial research was performed at Lawrence Berkeley, National Laboratory (LBNL) using neon-ions in 1957, but its medical program was closed in 1993 because of financial difficulty and aged machine.

In 1984 the heavy-ion therapy, project was started at NIRS as part of the national 10-year plan to combat cancer, and in 1994 clinical trials were begun using carbon-ions generated by the HIMAC (Heavy-Ion Medical Accelerator in Chiba), the world's only heavy-ion accelerator dedicated to medical use in a hospital environment. The clinical trials are primarily based on a toxicity, study to investigate radiation morbidity in normal tissues as well as to search for optimal dose-fractionations for tumor control. The RBE value of carbon-ion beams was found to be 3.0 for mouse skin reaction at the distal part of the peak. Through February 1997, a total of 230 patients were treated in dose-escalating Phase I/II studies for various type of tumors, including 43 patients with non-small cell lung cancers who completed the planned treatment and were followed-up for 6 months or more. Five patients had Stage IIIA tumors and 38 patients (39 tumors) had Stage I tumors. Thus far, two patient developed Grade 3 acute reactions, which were improved satisfactorily in response to
conservative steroid treatment, but none of the other patients has experienced any major radiation morbidity. The overall local control rates at 12 months following RT were 62% (16/26) for Stage I tumors and 67% (2/3) for Stage IIIA tumors. There appeared to be a dose-response correlation in local control of tumors: 40% (2/5) for 59.4 GyE, 50% (2/4) for 64.8 GyE and 71% (12/17) for 72.0 GyE. Regarding tumor response by pathological type, adenocarcinomas appeared to require higher dose for tumor control than squamous cell carcinomas. It is our preliminary judgment that carbon-ion therapy would provide improved local control in lung cancer, especially for those with adenocarcinomas.

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Gene Therapy of Lung Cancer in Japan: an Update and Future Expectations

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Lung cancer is a worldwide increasingly common disease for which conventional therapies are generally ineffective. It remains the leading cause of cancer death for men in Japan. Approaches at the molecular level have demonstrated that one of the mechanisms of human lung cancer development is genetic abnormalities that induce inactivation of tumor-suppressor activity. The \( p53 \) gene, whose normal role is to regulate cell cycle, apoptotic cell death, DNA repair, and many processes of gene transcription, is the most common altered gene yet described in human cancers including lung cancer. These findings suggest that introduction of the wild-type \( p53 \) gene could reverse the functional defects in lung cancer, thus having a therapeutic effect.

The efficacy of the \( p53 \) gene therapy protocols using replication-deficient viral vectors is now being evaluated in the US clinical trials. Tumor regression as well as tumor growth stabilization were noted, suggesting favorable responses in some patients. The trial will be expanded as a global study to assess the therapeutic feasibility in more patients; therefore, a protocol has been submitted to the Japanese review committee. We will be able to start a phase I trial for advanced lung cancer patients in the near future. However, the currently available viral vector-mediated delivery system has significant limitations. For example, the local intratumoral administration of vectors can not introduce exogenous \( p53 \) gene into 100% of cells consisting of tumor tissues and can not be expected to exhibit the antitumor activity at the distant metastatic lesions. Our research results have recently demonstrated that novel antiangiogenic properties induced by the wild-type \( p53 \) gene transfer may be involved in the antitumor effect, especially the bystander effect, of \( p53 \) gene therapy. The finding suggests the importance of basic research to overcome the
limitations. Moreover, in our trial, local effects such as the improvement of obstructive pneumonia as well as the relief of localized pain must be promptly evaluated as an endpoint. These efforts will improve the p53 gene therapy to be the standard therapy for lung cancer in the future.

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