Journal of Cellular Biochemistry Supplements 28/29:64–73 (1997)

Genetic Markers for Early Detection of Lung Cancer and Outcome Measures for Response to Chemoprevention

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Abstract Lung cancer is one of the leading causes of cancer death in the world. The high mortality rate for lung cancer probably results, at least in part, from the absence of standard clinical procedures for diagnosis of the disease at early and more treatable stages compared to breast, prostate, and colon cancers. The delineation of genetic alterations that occur in lung tumorigenesis may aid in both developing molecular markers for early detection and predicting of response to chemoprevention/chemotherapy. Cytogenetic and molecular genetic studies have shown that mutations in protooncogenes and tumor suppressor genes (TSGs) are critical in the multi-step development and progression of lung tumors. Inactivation of TSGs are by far the most common mutational events documented during the development of lung cancer. For example, loss of function of the Rb and/or p53 genes has been detected in both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). In addition, allelic loss analyses have implicated the existence of other tumor suppressor gene loci on 9p as well as on 3p, 5q, 8p, 9q, 11p, 11q, and 17q. We examined the short arm of chromosomes 3 and 9 for TSG loci by analyzing 23 squamous cell carcinomas of the lung with numerous microsatellite markers. On chromosome 9p, loss of heterozygosity was detected in all of the 23 tumors and homozygous deletions of the p16/CDKN2 locus were detected in 6 of the 23 (26%) tumors. In addition, a novel region of homozygous deletion was detected in 6 of the tumors (26%) at D9S126. The homozygous deletion of D9S126 was confirmed by fluorescent in situ hybridization (FISH) analysis of tumor tissue touch preparations and isolated nuclei using P1 and cosmid probes that contain D9S126. Only one tumor harbored a homozygous deletion at both the p16/CDKN2 locus and the D9S126 locus. The data identify a region of homozygous loss on the short arm of chromosome 9, suggesting the presence of a novel TSG locus approximately 2.5 cM proximal to p16/CDKN2. On chromosome 3p, a similar high percentage of the tumors exhibited loss of heterozygosity. Also, homozygous deletions were detected in several tumors at 3p21.3. Thus, FISH analysis with probes containing the D9S126 or p16 locus could be used as molecular markers to assay sputum samples for premalignant cells exfoliated from the bronchial epithelium. Probes from other chromosome regions such as 3p21 could be used in a similar manner. J. Cell. Biochem. Suppls. 28/29:64–73.

Key words: lung cancer; chemoprevention; genetic alterations; sputum cytology

After heart disease, cancer is the most common cause of death, and lung cancer is the most common cause of cancer mortality in the United States. From 1950–1988, lung cancer experienced the largest increase in mortality rate of all the cancers [1]. In 1996, an estimated 177,000 new cases and 158,700 deaths due to lung cancer occurred in the United States [2]. The prognosis is very poor for patients whose tumors cannot be completely resected, with almost 90% of the patients dying from the disease within 2 years of diagnosis [3]. Improvements in early detection, coupled with identification of a gene or genes that predispose individuals to lung cancer, could help reduce the death rate for lung cancer. As in other common malignancies such as breast, prostate, and colon cancer, patients whose lung cancer is detected early (small volume, localized, and non-metastatic) have the best response to known therapies and show improved long-term survival compared to patients with advanced stage disease [4,5].

Four major histological types of human lung cancer are adenocarcinoma, squamous cell car-

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Received 1 August 1997; Accepted 2 December 1997

cinoma, large cell carcinoma, and small cell carcinoma. Adenocarcinoma, squamous cell carcinoma, and large cell carcinoma are classified as "non-small cell lung cancer" (NSCLC). Recently, the proportion of lung tumors diagnosed as adenocarcinoma has increased, making it the most commonly occurring lung tumor in many parts of the world, accounting for 40% of newly reported primary lung cancer cases [6–11]. Adenocarcinoma is either solid or bronchioloalveolar. The overall increase in adenocarcinoma is due in part to an increase in bronchioloalveolar lung cancer (BAC). There are also three histologic subtypes of BAC (mucinous, non-mucinous, and sclerotic) that have distinct clinical behavior [6].

Lung carcinogenesis is the result of a series of genetic mutations that accumulate progressively in the bronchial epithelium, first generating histologically identifiable premalignant lesions and finally resulting in an invasive carcinoma. The premalignant genetic changes may occur many years before the appearance of invasive carcinoma. Morphologic analysis can identify several stages of premalignant lesions in the development of squamous cell carcinoma that arise in the bronchi [12–14]. The spectrum of histologic changes in prenoplastic bronchial epithelium extends from basal cell hyperplasia through squamous cell metaplasia to mild, moderate, and marked dysplasia, carcinoma in situ, and finally invasive cancer. More recently, Nagamoto et al. 1993 [15] suggested that squamous cell carcinoma of the bronchus may also develop from marked atypical basal cells in a pathway distinct from the one described above. In contrast, the development of an adenocarcinoma is not as clearly defined morphologically [7,16]. Microscopic serial section analysis of the tracheal bronchial trees from patients with both squamous cell and adenocarcinoma reveals multiple foci of basal cell hyperplasia. Whether or not these hyperplastic foci have the potential to develop into premalignant lesions and subsequently adenocarcinoma is presently not known; however, it has been suggested that atypical bronchioloalveolar hyperplasia may be a precancerous lesion that can transform into an adenocarcinoma [17,18]. Premalignant lesions are frequently observed not only in the tissue that surrounds the invasive carcinoma but also in regions distal to the tumor. Also, these premalignant lesions are frequently observed and collected during a bronchoscopic examination of high-risk patients prior to lung tumor diagnosis.

Two distinct theories have been proposed to explain the simultaneous and/or sequential development of multiple foci of preinvasive lesions as well as the occurrence of multiple primary and secondary tumors in the aerodigestive epithelium. The monoclonal neoplasia theory proposes that progeny from a single transformed cell can spread to produce multiple preinvasive lesions and tumors. The second theory holds that the continued exposure of the aerodigestive epithelium to carcinogens in tobacco smoke predispose the entire epithelium to develop multiple, independent preinvasive lesions that can develop into tumors. This concept is referred to as "field cancerization," which implies that the entire epithelium has been mutagenized. Several recent studies that examined mutations of the p53 gene and/or loss of heterozygosity (LOH) on 3p or 9p suggest that the multiple foci of preinvasive lesions and multiple primary tumors arise by the process of field cancerization [19–25]. Our data showing LOH on chromosome 9 for preinvasive lesions and tumors in the same patient also demonstrate the field cancerization phenomenon in the bronchial epithelium (unpublished data). In contrast, several studies suggest that multifocal tumorigenesis in the urothelium results from intraepithelial spreading of a single transformed cell [26–28].

In this report, we will discuss how a combination of molecular markers, sputum cytology, and bronchoscopy/CT may enhance early lung cancer detection and monitor the efficacy of chemoprevention agents.

Genetic Alterations in Lung Tumorigenesis

Cytogenetic and molecular studies have shown that mutations in protooncognes and tumor suppressor genes (TSGs) are critical in the multi-step development and progression of lung tumors [29,30]. Mutations in the K-*ras* protooncogene occur in 30–50% of pulmonary adenocarcinomas [31–34] and overexpression and/or amplification of Cyclin D1 [35] and the *myc* family of protooncogenes [36–39] have also been implicated in lung cancer. However, inactivation of TSGs is the most common event contributing to lung tumorigenesis.

Several well-characterized TSGs have been observed to be mutated in human lung tumors. Mutations of the Rb gene are almost always

present in SCLC, whereas the incidence in NSCLC ranges from 25–60% [40–42]. Mutations of the p53 gene have been observed in numerous human tumors, including lung with 70% of SCLC, 45% of squamous cell carcinoma, and 33% of adenocarcinomas containing a mutated p53 [43,44]. A recently identified cyclin-dependent kinase inhibitor, p16/CDKN2, has proven to be inactivated in several tumor types, including lung (see discussion below).

In addition to Rb, p53 and p16/CDKN2, allele loss analyses have implicated the presence of other TSG loci involved in lung tumorigenesis, including loci in 1p, 1q, 3p, 5q, 6p, 6q, 8p, 9p, 9q, 11p, 11q, 17p, 17q, 19p, and 21q (Table I). Some of these chromosomal regions are probably more important than others. In particular, specific loci on 3p and 9p are targets for deletion in a high percentage of lung tumors [45–49].

Three distinct regions on 3p (3p25, 3p21.2, 3p14-cen) appear to be targets for deletion in a high percentage of both SCLC and NSCLC [45]. Several LOH studies have suggested the involvement of 3p in all SCLC and in 27 to 100% of NSCLC [45, 50–52]. A TSG has been identified in 3p25–26 that is associated with the Von-Hippel-Lindau (VHL) familial cancer syndrome [53,54] and this gene may also be involved in lung cancer. However, Waber et al., 1996 [55] tested 26 matched normal and tumor samples from aerodigestive tract squamous cell carcinomas for mutations in the VHL gene. Although the authors were able to detect LOH in this region of all the samples, they were unable to detect mutations or methylation inactivation of this gene in any of the tumors. These results suggest that another TSG that is important in lung tumorigenesis may be located in this region. Two of the three DNA repair genes, HHR23A and XPC, involved in xeroderma pigmentosum subgroup C, have also been localized to band 3p25.1 by in situ hybridization and may be candidate TSGs. Pulsed field gels revealed that these genes may be contained within a region of 625 kb [56]. The protein tyrosine phosphatase gene [57] and a mitogen-activated protein kinase (3pK) [58] are candidate TSGs located on 3p21. We have also recently demonstrated two regions of homozygous deletion, one at 3p21 and the other at 3p12 [46]. A putative TSG is currently being mapped to a region near a fragile site at 3p14.2 and several TSGs have been identified in this region of deletion. These include the FHIT gene [59], a member of the

TABLE I. Tumor Suppressor Genes Implicated in Human Lung Cancer

Chromosomal arm	Identified gene, chromosomal region, or specific locus	References
3p	FHIT,[a] VHL,[b] 3p25, 3p21.3, and 3p14	Hibi et al. [45] Todd et al. [46] Sozzi et al. [19] Wei et al. [87]
9p	p16/CDKN2 and the D9S126 locus	Cairns et al. [72], Wiest et al. [49]
11p	11p13 and 11p15.5	Bepler & Garcia-Blanco [88]
11q	Between D11S940 and CD3D, between D11S924 and D115925, and between D1151345 and D1151328	Rasio et al. [89]
13q	RB	Reissmann et al. [90]
17p	p53 and a region distal to p53	Greenblatt et al. [44] Wales et al. [91]
17q	Between D17S40 and D17S21 and a region telomeric to BRCA1	Fong et al. [92]
9q	9q22.3	Merlo et al. [47, 48] Testa et al. [93]
5q	APC/MCC region at 5q21 and more telomeric region at 5q33-35	Hosoe e tal. [94] Wieland & Bohm [95]
6p		Merlo et al. [48]
6q	6q24-25[a]	Merlo et al. [48] Testa et al. [93]
8p	8p21.3 between the markers C18-1051 and C18-2644	Emi et al.[96] Fujiwara et al. [97] & Testa et al. [93]
1p		Testa and Siegfried [62] Lukeis et al. [61]
1q		Tsuchiya et al. [98]
19p		Lukeis et al. [61] Testa et al. [93]
21q		Testa et al. [93] Sato et al. [40]

[a]FHIT is located at 3p14.2. However, the extent of involvement of this gene in lung cancer is unclear at present.
[b]VHT is located at 3p25. This gene is only rarely mutated in primary lung cancer.
[c]Tumor suppressor gene(s) have been suggested to reside in this region for ovarian and breast cancer.

semaphorin genes [60], and a member of the human G protein alpha i2. However, none of these genes has been confirmed as tumor suppressor genes.

There is considerable cytogenetic evidence for genetic alterations on chromosome 9p [61,62]. Olopade et al., 1993 [63] used molecular probes to loci on 9p to show that NSCLC cell lines have loss of DNA sequences on 9p, which includes the interferon genes. More recently, Merlo and coworkers used polymorphic microsatellite markers to detect LOH at loci on 9p in 67% of NSCLC [47] and 52% of small cell lung tumors [48]. The minimal area of loss on chromosome 9p in the NSCLC was mapped distally to the interferon-alpha (IFNA) marker and proximally to the D9S171 marker covering approximately a 2-cM region. Deletions in this same region of 9p have also been observed in numerous other tumors, including head and neck squamous cell carcinomas [56], bladder tumors [64], melanomas [65], mesotheliomas [66], gliomas [67], and nasopharyngeal carcinomas [68]. The p16/CDKN2 tumor suppressor gene is located in 9p21 [69]; a high percentage of alterations in this gene have been observed in some of the tumor types with LOH on 9p, including esophageal tumors, pancreatic adenocarcinomas [70], glioblastomas [71], and bladder tumors [72]. In contrast, the frequency of mutations observed in p16/CDKN2 in human lung tumors is lower than the frequency of LOH on 9p [72,73]. Moreover, deletion mapping studies of various tumor types suggest the existence of more than one tumor suppressor gene on 9p [74–76]. Also, we have recently demonstrated a region of homozygous deletion in squamous cell carcinomas of the lung at 9p21 that may harbor a novel tumor suppressor gene [49]. Thus, in addition to p16/CDKN2, another tumor suppressor gene(s) may reside on 9p that contributes to the development of lung and other tumor types.

Our allelic loss analyses on 3p and 9p from a set of 23 squamous cell carcinomas of the lung [46,49] were the first to extensively examine loss of heterozygosity (LOH) on these chromosome arms in the same set of tumors. A high percentage of the tumors exhibited LOH at both 3p and 9p. Twenty-two of the 23 tumors had allelic loss at more than one informative locus on 9p; the other tumor showed LOH only at D9S162. Ten of the tumors had LOH at all informative markers. Four others had LOH at all but one informative marker. Also, specific loci exhibited LOH at a high frequency. For example, D9S126 had LOH in 22 of 23 tumors, the p16/CDKN2 locus also had allelic loss in 22 of 23 tumors, and both loci had LOH in 20 of the 23 tumors. Two regions of homozygous loss were detected on 9p in the squamous cell tumors [49]. One region contains the p16/CDKN2 TSG and the other region is proximal to p16/CDKN2 and located between D9S265 and D9S259. The p16/CDKN2 had previously been shown to be homozygously deleted in primary lung tumors [72]. However, homozygous deletions at the D9S126 locus had not been previously observed in primary tumors; thus, this region may harbor a novel TSG. Homozygous deletions were observed in six tumors for both the D9S126 locus and in six tumors for the p16/CDKN2 locus. However, only one tumor had a homozygous deletion at both of these loci (Table II). The homozygous deletions at D9S126 were confirmed by fluorescent in situ hybridization (FISH) analyses using a P1 probe containing the D9S126 locus.

Similarly, extensive LOH was observed on 3p in these tumors. Twenty of the 23 tumors had allelic loss at more than one locus. Twelve of the tumors exhibited LOH at all informative markers on 3p. It is very probable that at least two TSGs are inactivated on each of 3p and 9p in a large majority of human lung tumors. Table II summarizes our allelic loss data on 3p and 9p, as well as mutation analysis of the p53 gene, for each of the 23 tumors. Ten of the 23 tumors contained a p53 mutation. Homozygous deletions were detected in three of the squamous cell carcinomas within a region of 3p21 that had previously been described only in cell lines [46]. FISH analysis with probes containing TSGs on 3p, 9p, or other chromosomal regions can be used to assay sputum samples for premalignant cells exfoliated from bronchial epithelium.

Deletion of critical regions of chromosomes 3p and 9p has been demonstrated in premalignant lesions of the lung [23,24,77], oral cavity [78], and head and neck [25]. The data presented in Table III suggest that deletions on the short arm of chromosomes 3 and 9 are important early events in lung tumorigenesis and other cancer types arising from intraepithelial lesions. In general, the incidence of these deletions increases as histopathological lesions advance from hyperplasia to dysplasia to CIS. Also, allelic loss was observed at both 3p and 9p

TABLE II. Summary of Chromosome 9p and 3p Allelic Loss Analyses and p53 Mutations in Squamous Cell Carcinomas of the Lung*

Tumor no.	Tumor stage	Chromosome 9p	Chromosome 3p	p53 Mutation
22	I	HD of D9S126	Large deletion	Exon5
23	I	HD of p16	HD of D3S2968	
27	I	Loss of 9p	Large interstitial deletion	Exon5
30	I	HD of D9S126	Loss of 3p	
50	I	Large deletion	Loss of 3p	
51	I	Loss of 9p	Loss of 3p	
52	I	HD of D9S126	Loss of 3p	
58	I	HD of D9S126	Loss of 3p	Exon5
61	I	LOH of D9S126	No loss	Exon7
64	I	HD of p16 & D9S126	Loss of 3p	
65	I	Loss of 9p	Large interstitial deletion	Exon8
66	I	Loss of 9p	Loss of 3p	
26	II	HD of p16	HD of D3S2968	
39	II	Large deletion	Loss of 3p	
62	II	Loss of 9p	No loss	Exon5
63	II	Large deletion	Loss of 3p	
24	IIIA	HD of D9S126	No loss	Exon7
37	IIIA	Loss of 9p	Large interstitial deletion	
43	IIIA	HD of p16	Loss of 3p	Exon5
44	IIIA	HD of p16	Large Telomeric deletion	Exon5
45	IIIA	HD of p16	Large deletion	
59	IIIA	Large deletion	Loss of 3p	
60	IV	LOH of D9S156 and p16	Loss of 3p	Exon5

*Data from 3p and 9p allelic loss analyses from references [44, 47]. HD: homozygous deletion; p16: p16/CDKN2 gene.

in some of the prenoplastic lesions and was more frequent in the later stage lesions; for example, both 3p and 9p deletions were detected in 0/7 hyperplasia, 1/7 mild dysplasia, 7/1/7 moderate dysplasia, and 3/4 CIS [77]. In addition, Mao et al. [78] reported that 7 of 19 patients with LOH of at least one marker on either 3p or 9p went on to develop head and neck squamous cell carcinomas; only 1 of 18 patients without detected LOH developed tumors. These results are consistent with the observation that allelic losses on 3p and 9p are detected in a high number of these types of genetic alterations in premalignant lesions, and may serve as markers of risk and aid in monitoring of chemopreventive trials.

Early Detection of Lung Cancer and Outcome Measures for Response to Chemoprevention

Lung cancer is one of the most lethal types of cancer to acquire, as reflected in a 5-year survival rate of only 14% [2]. The poor prognosis for lung cancer patients is due, in part, to the historical lack of effective early detection measures. At the time of clinical presentation, over two-thirds of the patients have clinically detectable regional nodule involvement or distant metastases, both of which are usually incurable by systemic therapy [79,80]. The Surveillance, Epidemiology and End Results Reporting (SEER) Program of the National Cancer Institute (NCI) [81] found that individuals with "localized" (confined to the site of origin) lung cancer treated by surgical resection had a 50%, 5-year survival rate. Other studies have reported similar results in patients diagnosed early with stage I tumors, with 5-year survival rates ranging between 40–70% following resection [4,79,82]. These observations support efforts to develop better methods for early lung cancer detection, under the assumption that such detection will lead to diagnosis at an earlier stage more amenable to potentially curative treatment.

Two studies suggest that a majority of patients presenting with a positive sputum cytologic test and a negative chest X ray will survive their cancer at least 5 years [83,84]. In the study of Saito and colleagues, 1992 [83], impressive results were achieved in 94 patients with X ray-negative, squamous cell carcinoma who underwent surgical resection. The 5-year survival

TABLE III. Allelic Loss Analysis on 3p and 9p in Pneneoplastic Epithelial Lesion of Lung, Head and Neck, and Oral Cavity

Tissue	Premalignant epithelial lesion	Allelic loss[a]		
		3p14	3p21	9p21
Lung[b]	Hyperplasia	6/16 (35)	10/28 (36)	5/27 (19)
	Dysplasia	2/7 (29)	15/36 (42)	9/25 (36)
	CIS	3/3 (100)	10/10 (100)	8/11 (73)
Head and neck[c]	Hyperplasia		5/31 (16)	8/25 (20)
	Dysplasia		15/29 (52)	17/30 (57)
	CIS		12/20 (60)	17/21 (80)
Oral cavity[d]	Hyperplasia	9/52 (17)		12/52 (23)
	Dysplasia	6/32 (19)		12/32 (38)
	CIS			

[a]No. of lesions with allelic loss/no. of informative lesions (%).
[b]Data from Kishimoto et al. [23], Thiberville et al. [77], and Hung et al. [24].
[c]Data from Califano et al. [25].
[d]Data from Mao et al. [78].

rate, including deaths from all cancer, was 80.4%; the 5-year survival involving deaths only from lung cancer was 93.5%. Seventy-five patients with intrabronchial cancer without lymph node metastasis had complete resection with no local recurrence or metastasis of lung cancer. Fifty-three of the patients had no clinical symptoms at the time of diagnosis by sputum cytology, while others presented with cough and/or production of sputum. Similar results were obtained by Bechtel and associates in 1994 in 51 subjects with sputum-positive, roentgenographically occult lung cancer [84]. In this study, 27 patients underwent surgery and 19 were treated with radiation therapy. Of the 46 patients who received theraputic intervention, 9 lung cancer deaths and 21 deaths from all causes occurred within 5 years post-treatment; the actuarial survival of the study population, including deaths from all causes, was 55%.

In the Japanese study, cancer was detected in 27 of the patients based on a sputum diagnosis of borderline atypical squamous cells (terminology used in reference [83]). Of the 197 patients who exhibited borderline atypical squamous cells by sputum cytology, bronchoscopic examination detected squamous cell carcinoma in 27 of the cases. These authors concluded that bronchoscopic examination is essential in patients for whom sputum cytology study is suggestive but not conclusive for squamous cell carcinoma. It is possible that molecular markers such as LOH on 3p and 9p, mutant p53, etc., may be able to detect which of the borderline atypical squamous cells were exfoliated from a tumor. Based on the results, authors of both of these studies recommend periodic chest X-rays and sputum cytology exams for persons at high risk for developing lung cancer, including heavy smokers and/or individuals with increased occupational, environmental, or genetic risk [83,84]. However, these studies only detected squamous cell carcinomas at early stages; when combining the data of Saito et al. 1992 [83] and Bechtel et al., [84], over 95% of the lung tumors diagnosed in patients with positive sputum cytology and negative chest X-rays were squamous cell carcinomas.

Although sputum cytology has been shown to be reasonably effective in detecting squamous cell carcinoma, its diagnostic value declines drastically for adenocarcinoma [85]. The presence of adenocarcinoma cells in the sputum is a poor prognostic sign for patient survival. In a 1992 study by Miura and co-workers, adenocarcinoma cells were present in the sputum of 29 of 114 patients with adenocarcinoma who had sputum cytologic testing prior to bronchoscopy and surgical resection [86]. None of the patients with adenocarcinoma cells in the sputum survived 5 years. At present, the inability to detect adenocarcinoma at early stages by sputum cytology may be due to the absence of morphologic criteria to discriminate exfoliated atypical cells from premalignant lesions at risk of developing into adenocarcinomas [7,16]. Also, the localization of small, early stage adenocarcinomas by conventional bronchoscopy is problematic, since this histologic subtype tends to arise in the periphery of the lung. Adenocarcinomas now represent one of the most common forms of lung neoplasm, accounting for 40% of newly re-

ported primary lung cancer cases [6–11]. Thus, developing procedures to detect and localize this histologic subtype will help reduce the mortality rate of lung cancer.

In summary, the most promising approach to improving the frequency of early lung cancer detection in high-risk patients and to monitor the efficacy of chemoprevention protocols is to combine several procedures/techniques in a systematic manner. First, the non-invasive procedure of collecting adequate sputum samples should be followed by both sputum cytology and the use of molecular markers to detect premalignant cells exfoliated from stage I tumors or advanced stage premalignant lesions, e.g., severe dysplasia, CIS, or atypia basal cell hyperplasia [15–18]. If "suspicious cells" are detected, then bronchoscopy and/or helical CT scan can be employed to localize the early stage cancer or advanced stage premalignant lesion. These studies must be used in a longitudinal fashion in high-risk patients as mammography is used in early breast cancer diagnosis.

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