# Numerical Abnormalities of Chromosome 9 and *p16$^{CDKN2A}$* Gene Deletion Detected by FISH in Non-small Cell Lung Cancer

ANNA D. PANANI[1], KATERINA MALIAGA[1], ATHANASIA BABANARAKI[1] and ION BELLENIS[2]

*[1]Critical Care Department, Medical School of Athens University, Cytogenetics Unit, and [2]Department of Thoracic and Vascular Surgery, Evangelismos Hospital, Athens, Greece*

**Abstract.** *Background: Lung cancer is one of the most common types of cancer worldwide and its pathogenesis is closely associated with various environmental exposures and gene alterations. The identification of genetic changes is a useful strategy toward understanding tumourigenesis and specific genetic associations. Since the tumor suppressor gene p16 located at 9p21 chromosomal region might have an important role in lung carcinogenesis, the aim of the present study was to investigate p16 gene alterations and numerical aberrations of chromosome 9 in non-small cell lung cancer. Materials and Methods: Nineteen cases of non-small cell lung cancer (11 squamous cell carcinomas, 6 adenocarcinomas and 2 large cell carcinomas) were investigated by fluorescence in situ hybridization (FISH) technique using a DNA p16 probe and α-satellite probe specific for chromosome 9. Results: Polysomy 9 was found in 13 cases (6/11 squamous cell carcinomas, 5/6 adenocarcinomas and 2/2 large cell carcinomas). p16 gene alterations were found in 16 cases. Among them, deletion of p16 gene was found in 15 cases (8/11 squamous cell carcinomas, 5/6 adenocarcinomas and 2/2 large cell carcinomas). In six cases with p16 gene deletion, homozygous deletion was observed. Conclusion: Numerical aberrations of chromosome 9 and p16 gene deletion are common findings in all subtypes of non-small cell lung cancer. Despite suggesting the p16 gene in the 9p chromosomal region plays a role in lung carcinogenesis, the presence of other oncogenes reflected by polysomy 9 participating in the neoplastic process cannot be excluded. Data of the present study also suggest, that there might not be a fundamental relationship between genetic changes and histological subtype of non-small cell lung cancer.*

*Correspondence to:* Anna D. Panani, Critical Care Department, Medical School of Athens University, Evangelismos Hospital, Ipsilandou 45-47, Athens 106 76, Greece. Tel: +30 2107259307, Fax: +30 2107259307, e-mail: apanani@med.uoa.gr

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Lung cancer is one of the most common types of cancer worldwide and its pathogenesis is closely associated with various environmental exposures and gene alterations. There are poor informative data regarding the sequence of genetic changes leading to lung cancer development. Several reports suggested that a number of molecular genetic and epigenetic events contribute to lung cancer development, while other studies have investigated the use of certain genetic alterations as potential biomarkers in early detection or risk assessment of lung cancer (1-9).

The tumor suppressor gene *p16$^{CDKN2A}$* seems to have an important role in lung carcinogenesis. It is located at 9p21 and encodes a cell cycle protein that is an inhibitor of cyclin dependent kinases (CDK) 4 and 6 and negatively regulates cyclin D-dependent phosphorylation of the *Rb* gene product, thus blocking cell cycle progression from the $G_1$- to the S-phase. Loss of function of *p16* gene has been reported to occur mainly by homozygous deletions, mutations or aberrant DNA methylation of the promoter region (1, 10-13).

The aim of the present study was to investigate both numerical aberrations of chromosome 9 and *p16* gene alterations in surgically resected tumors of non-small cell lung cancer (NSCLC) by fluorescence *in situ* hybridization (FISH) technique.

## Materials and Methods

Reviewing cancerous tumors cytogenetically studied in our laboratory, 51 patients with NSCLC were found who had undergone surgical resection of the tumors between the years 2004 and 2005. Since the *p16* gene is considered to play an important role in lung carcinogenesis, among methods used for cytogenetic evaluation of the cases studied, FISH technique using a DNA *p16* probe had been

Table I. *Numerical aberrations of chromosome 9 and p16 gene alterations in 19 cases of non-small cell lung cancer.*

| Case no. | Histological type | Chromosome 9 numerical aberrations | | | Alterations of *p16* gene | | | |
|---|---|---|---|---|---|---|---|---|
| | | Monosomy (%)* | Disomy (%)* | Polysomy (%)* | Deletion (red<green spots) (%)* | Deletion (1 red /1 green spot) (%)* | Gains of *p16* copies equal to chromosome 9 copies (%)* | None (2 red/2 green spots) (%)* |
| 1 | Squamous | 3.00 | 91.52 | 5.48 | 19.20 | 2.30 | 1.55 | 76.95 |
| 2 | Large cell | 3.50 | 37.40 | 59.1 | 33.91 | 2.60 | 34.69 | 28.80 |
| 3 | Squamous | 2.77 | 91.36 | 5.87 | 2.43 | 2.46 | 3.65 | 91.46 |
| 4 | Squamous | 36.44 | | 63.56 | 6.20 | 36.44 | 57.36 | |
| 5 | Squamous | 22.94 | 10.09 | 66.97 | 66.97 (62.38)** | 22.94 | | 10.09 |
| 6 | Squamous | | 31.07 | 68.93 | 68.93 (65.04)** | | | 31.07 |
| 7 | Squamous | | 99.04 | 0.96 | 7.69 | | 0.96 | 91.35 |
| 8 | Squamous | 2.63 | 86.85 | 10.52 | 38.59 (24.56)** | 2.63 | 1.77 | 57.01 |
| 9 | Squamous | | 98.30 | 1.70 | 32.88 | | | 67.12 |
| 10 | Squamous | | 46.62 | 53.38 | 41.52 | | 11.86 | 46.62 |
| 11 | Adenocarcinoma | | 83.05 | 16.95 | 37.29 | | 3.39 | 59.32 |
| 12 | Adenocarcinoma | 5.88 | 74.32 | 19.80 | 68.63 | 5.87 | 3.93 | 21.57 |
| 13 | Large cell | | 71.21 | 28.79 | 43.94 (41.67)** | | | 56.06 |
| 14 | Squamous | | 52.06 | 47.94 | 51.24 | | | 48.76 |
| 15 | Squamous | 2.90 | 93.80 | 3.30 | 85.40 (51.40)** | 2.9 | 0.5 | 11.20 |
| 16 | Adenocarcinoma | | 16.00 | 84.00 | 85.00 | | | 15.00 |
| 17 | Adenocarcinoma | 0.87 | 35.65 | 63.48 | 9.57 | 0.87 | 53.91 | 35.65 |
| 18 | Adenocarcinoma | 2.00 | 76.00 | 22.00 | 35.00 (21.00)** | | 11.00 | 54.00 |
| 19 | Adenocarcinoma | | 92.83 | 7.17 | 11.60 | | 1.80 | 86.60 |

*Of total cells examined; **cells with homozygous deletion.

performed in 19 lung cancer cases. These cases were included in the present study. Eleven cases had squamous cell carcinoma of low differentiation, six cases had adenocarcinomas, while two cases were large cell carcinomas. Tissue specimens were collected from fresh surgically resected tumors. None of the patients had ever received chemotherapy or radiation prior to surgery. Further clinical data regarding tumor biological behavior or disease outcome were not available for the cases studied. A small portion of each resected tumor was directly processed for cytogenetic study as described elsewhere (14). FISH technique was applied to recently made slides from methanol/acetic acid-fixed cells using a DNA *p16* probe and α-satellite probe specific for chromosome 9. The *p16* probe (Cytocell Ltd, Cambridge UK), labeled red, covered a 101 kb region of 9p21, extending from 59 kb 3' of *p16* to the 5' end of p15. The probe mix also contained a control probe for chromosome 9 (D9Z3, the hetechromatic block at 9q12), labeled green. FISH was carried out according to the manufacturer's instructions. The hybridization of the probe with the cellular DNA site was visualized by fluorescence microscopy using a NIKON E600 equipped with selective filters for the fluorochromes used. Cells with deletion of *p16* gene have either one red signal and two green controls if the deletion is hemizygous, or no red but two green signals if the deletion is homozygous. A minimum of 200 non-overlapping cells from each slide were evaluated for each case. Signals were scored using the criteria of Hopman *et al*. (15). To avoid misinterpretation due to technical error, normal lymphocyte nuclei were used as a control. Approximately 96% of control lymphocyte nuclei showed two red signals for *p16* gene and two green signals for the 9q12 chromosomal region. A case was counted as aberrant if more than 10% of the cell nuclei showed losses or gains of signals for chromosome 9 or *p16* gene. This study was approved by the local Ethical Committee.

## Results

Results are shown in Table I. Numerical aberrations of chromosome 9 were found in 13 out of 19 cases studied (Figure 1); polysomy 9 was found in all 13 cases. Among them, 6 cases had squamous cell carcinomas, 5 cases adenocarcinomas and 2 cases large cell carcinomas. In 2 cases (cases 4 and 5) there were two cell populations, one with polysomy 9 and the other with monosomy 9. *p16* gene alterations were found in 16 cases. Among them, deletion of *p16* gene (red spots<green spots) was found in 15 cases (8 cases of squamous cell carcinomas, 5 cases of adenocarcinomas and 2 cases of large cell carcinomas). In one case of squamous cell carcinoma (case 4), 36.44% of the examined cells exhibited one red and one green spot. In addition, in this case, 57.36% of the examined cells presented gains of *p16* gene equal to chromosome 9 copies. In six cases with *p16* gene deletion, homozygous deletion was observed in 62.38, 65.04, 24.56, 41.67, 51.40 and 21.00% of the examined cells, respectively.

## Discussion

Several studies have investigated the genetic alterations in lung carcinogenesis in order to predict prognosis and develop new therapeutic strategies, but no specific molecular marker has as yet been defined in lung cancer. Molecular studies focusing on the tumor suppressor gene *p16* showed that *p16* inactivation has an important role in lung carcinogenesis. It was reported that *p16* gene is inactivated in up to 70% of NSCLC tumor specimens but rarely in small cell lung cancer (SCLC).The inactivation of *p16* is mainly caused by homozygous deletions, mutations or promoter hypermethylation of the gene. The rate of mutation of *p16* is relatively low in NSCLC, whereas homozygous deletions of the gene can be found in 10-40% of the tumors. It has also been reported that aberrant methylation of *p16* gene is an early event in lung cancer (1, 10, 16-20). Most of the studies regarding *p16* gene alterations were based on polymerase chain reaction (PCR) analysis techniques or immunohistochemistry for gene protein expression (11, 16-17, 20).

We utilized FISH to study numerical aberrations of chromosome 9 and *p16* gene alterations in NSCLC. The FISH technique is considered the most reliable method for detecting homozygous gene deletions. Numerical abnormalities of chromosome 9 were found in 13 cases whereas deletion of *p16* gene was observed in 15 cases. In one case (case 4) with cells exhibiting both monosomy and polysomy of chromosome 9, 36.44% of the examined cells exhibited one red and one green spot; moreover, 57.36% of the examined cells presented gains of *p16* gene equal to chromosome 9 copies. It seems evident, that in cases with 1 red and 1 green spot, loss of *p16* gene is due to chromosome 9 monosomy. Similarly, in cases with gains of *p16* gene copies equal to those of chromosome 9, the gains of *p16* gene copies result from chromosome 9 polysomy.

Dessy *et al*. (17) studied chromosome 9 and *p16* gene alterations by FISH technique and immunohistochemistry in the tumor specimens of 31 patients with squamous cell carcinomas and in 31 adjacent normal bronchi specimens. They found numerical aberrations of chromosome 9 in 19/31 patients, whereas *p16* gene alterations were present in 29/31 cases. In that study, among cases with *p16* gene alterations, 8 cases with polysomy of chromosome 9 also had gains of copies of *p16* gene. However, it was not clear in the above cases, whether the *p16* gene copies were equal to or fewer than the copies of chromosome 9. Moreover, 4 patients with monosomy of chromosome 9 had one copy of *p16* gene. The authors also concluded that inactivation of *p16* gene is an early event in the evolution of the bronchial epithelium towards carcinoma.

Regarding histological type, numerical aberrations of chromosome 9 were observed in 6/11 squamous cell carcinomas, 5/6 adenocarcinomas and in 2/2 large cell carcinomas. Deletion of *p16* gene was observed in 8/11 squamous cell carcinomas, in 5/6 adenocarcinomas and in 2/2 large cell carcinomas. Although, the number of cases studied was too small in order to detect any relationship between histopathological types of lung cancer and genetic changes by statistical analysis, it seems that numerical aberrations of chromosome 9 and *p16* gene deletion are shared by all histological subtypes of NSCLC.

Several studies have described common chromosomal abnormalities in lung cancer, but little is known about the mechanisms behind these chromosomal changes (21-23). Although chromosomal changes in lung carcinomas may be recurrent, they lack diagnostic specificity; they are considered part of a stepwise process facilitating the identification of genes important in carcinogenesis. Regarding chromosome 9 aberrations, besides the *p16* gene in the 9p chromosomal region, the presence of other oncogenes in concert with polysomy of chromosome 9 participating in the neoplastic process cannot be excluded.

Interestingly, in one study, all published cases of cytogenetically aberrant lung cancers in the Mitelman Database of Chromosome Aberrations in Cancer, 432 cases in total, were statistically analyzed to detect possible karyotypic pathways and possible cytogenetic subtypes (23). It was found that the profiles for adenocarcinomas, squamous and large cell carcinomas were very similar. On the other hand, *p16* gene alterations were described in several types of malignant diseases, including hematological malignancies (24). Regarding lung cancer, several studies have shown that *p16* inactivation occurred in all histological subtypes of NSCLC (16-17). Data of the present study also suggested that there might not be a fundamental relationship between genetic changes and any particular histological subtype of NSCLC. Therefore, the fact that similar recurrent genetic abnormalities have been observed within different histological subtypes of NSCLC, might suggest that there are no fundamental tissue-specific differences in the genetic changes by which lung neoplasia is initiated or progresses. However, in order to establish the above concept more cytogenetic information is needed.

In conclusion, FISH technique using the cocktail probes of α-satellite probe specific for chromosome 9 and a specific probe for *p16* gene is a valuable method for simultaneously detecting numerical abnormalities of chromosome 9 and *p16* gene deletion. Numerical aberrations of chromosome 9 and *p16* gene deletion are common findings in all subtypes of NSCLC, suggesting that there might not be a fundamental relationship between genetic changes and the histological subtype of NSCLC. Besides the *p16* gene on 9p chromosomal region being suggested as playing a role in lung carcinogenesis, the presence of other oncogenes in association with polysomy of chromosome 9 participating in the neoplastic process cannot be excluded. Genetic changes

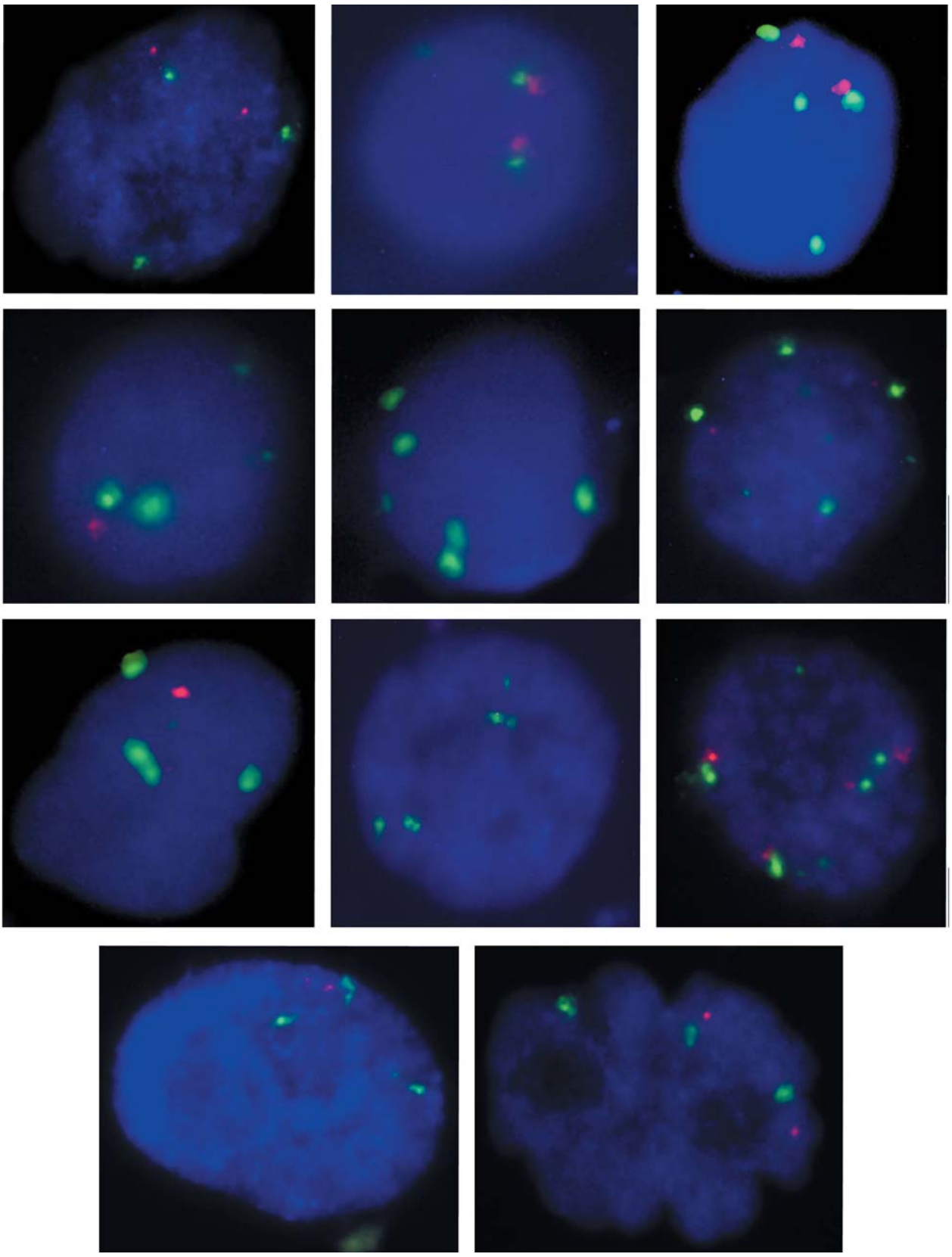

Figure 1. *Copy number of chromosome 9 (green spots) and p16 gene (red spots) detected by FISH in lung cancer cells from different cases.*

leading to lung cancer development or progression are of major importance and need to be thoroughly investigated, thus also contributing to the classification of this disease.

## References


1 Panani AD and Roussos C: Cytogenetic and molecular aspects of lung cancer. Cancer Lett *239*: 1-9, 2006.

2 Huber RM. And Stratakis DF: Molecular oncology-perspectives in lung cancer. Lung Cancer *45*: S209-S213, 2004.

3 Cheng S, Gao Y, Dong X, Lu Y, An Q, Tong T and Wang Y: Molecular and cytogenetic alterations in early stage of carcinogenesis of human lung. Cancer Lett *162*: S5-S10, 2001.

4 Wistuba I, Behrens C, Milchgrub S, Bryant D, Hung J, Minna JD and Gazdar AF: Sequential molecular abnormalities are involved in the multistage development of squamous cell lung carcinoma. Oncogene *18*: 643-650, 1999.

5 Mao L: Molecular abnormalities in lung carcinogenesis and their potential clinical implications. Lung Cancer *34*: 527-534, 2001.

6 Kettunen E, Anttila S, Seppanen JK, Karjalainen A, Edgren H, Lindstrom I, Salovaara R, Nissen A-M, Salo J, Mattson K. Hollmen J, Knuutila S and Wikman H: Differentially expressed genes in non-small cell lung cancer: expression profiling of cancer-related genes in squamous cell lung cancer. Cancer Genet Cytogenet *149*: 98-106, 2004.

7 Steels E, Paesmans M, Berghmans T, Branle F, Lemaitre F, Mascaux C, Meert AP, Vallot F, Lafitte JJ and Sculier JP: Role of *p53* as a prognostic factor for survival in lung cancer: a systematic review of the literature with a meta-analysis. Eur Respir J *18*: 705-719, 2001.

8 Huncharek M, Kuplnick B, Geschwind JF and Caubet JF: Prognostic significance of *P53* mutations in non-small cell lung cancer: a meta-analysis of 829 cases from eight published studies. Cancer Lett *153*: 219-226, 2000.

9 Yakut T, Egeli U and Gebitekin C: Investigation of *C-MYC* and *P53* gene alterations in the tumor and surgical borderline tissues of NSCLC and effects on clinicopathologic behavior: By the FISH technique. Lung *181*: 245-258, 2003.

10 Belinsky SA, Nikula KJ, Palmisano WA, Michels R, Saccomanno G, Gabrielson E, Baylin SB and Herman JG: Aberrant methylation of *P16*$^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci USA *95*: 11891-11896, 1998.

11 Mariatos G, Gorgoulis VG, Zacharatos P, Kotsinas A, Vogiatzi T, Rassidakis G, Foukas P, Liloglou T, Tiniakos D, Angelou N, Manolis EN, Veslemes M, Field JK and Kittas C: Expression of *p16*$^{INK4A}$ and alterations of the 9p21-23 chromosome region in non-small cell lung cancer carcinomas: relationship with tumor growth parameters and ploidy status. Int J Cancer *89*: 133-141, 2000.

12 Geradts J, Fong KM, Zimmerman PV, Maynard R and Minna JD: Correlation of abnormal *RB*, *P16*$^{INK4a}$ and *P53* expression with 3p loss of heterozygosity, other genetic abnormalities, and clinical features in 103 primary non-small cell lung cancers. Clin Cancer Res *5*: 791-800, 1999.

13 Liggett WH and Sidransky D: Role of the *p16* tumor suppressor gene in cancer. J Clin Oncol *16*: 1197-1206, 1998.

14 Stamouli M, Ferti AD, Panani AD, Raftakis J, Consoli C, Raptis SA and Young BD: Application of multiplex fluorescence *in situ* hybridization in the cytogenetic analysis of primary gastric carcinoma. Cancer Genet Cytogenet *135*: 23-27, 2002.

15 Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Deville P, Ploeg M and Vooijs GP: *In situ* hybridization as a tool to study numerical aberrations in solid bladder tumors. Histochemistry *89*: 307-316, 1988.

16 Tanaka R, Wang D, Morishita Y, Inadome Y, Minami Y, Iijima T, Fucai S, Goya T and Noguchi M: Loss of function of *p16* gene and prognosis of pulmonary adenocarcinoma. Cancer *103*: 608-615, 2005.

17 Dessy E, Rossi E, Berenzi A, Tironi A, Benetti A and Grigolato P: Chromosome 9 instability and alterations of *p16* gene in squamous cell carcinoma of the lung and in adjacent normal bronchi: FISH and immunohistochemical study. Histopathology *52*: 475-482, 2008.

18 Kraunz KS, Nelson HH, Lemos M, Goldleski JJ, Wiencke JK and Kesley KT: Homozygous deletion of *p16*$^{INK4a}$ and tobacco carcinogen exposure in non-small cell lung cancer. Int J Cancer *118*: 1364-1369, 2006.

19 Nakata S, Sugio K, Uramoto H, Oyama T, Hanagiri T, Morita M and Yasumoto K: The methylation status and protein expression of *CDH1, p16*$^{INK4A}$ and fragile histidine triad in non-small cell lung canrcinoma. Cancer *106*: 2190-2199, 2006.

20 Jin M, Inoue S, Umemura T, Moriya J, Arakawa M, Nagashima K and Kato H: *Cyclin D1, P16* and retinoblastoma gene product expression as a predictor for prognosis in non-small cell lung cancer at stages I and II. Lung Cancer *34*: 207-218, 2001.

21 Balsara BR and Testa JR: Chromosomal imbalances in human lung cancer. Oncogene *21*: 6877-6883, 2002.

22 Pei J, Balsara BR, Li W, Litwin S, Gabrielson E, Feder M, Jen J and Testa JR: Genomic imbalances in human lung adenocarcinomas and squamous cell carcinomas. Genes Chromosome Cancer *31*: 282-287, 2001.

23 Hoglund M, Gisselsson D, Hansen GB and Mitelman F: Statistical dissection of cytogenetic patterns in lung cancer reveals multiple modes of karyotypic evolution independent of histological classification. Cancer Genet Cytogenet *154*: 99-109, 2004.

24 Tsirigotis P, Pappa V, Labropoulos S, Papageorgiou S, Kontsioti F, Dervenoulas J, Papageorgiou E, Panani A, Mantzios G, Economopoulos T and Raptis S: Mutation and methylation analysis of cyclin-dependent kinase 4 inhibitor (*p16*$^{INK4A}$) gene in chronic lymphocytic leukemia. Eur J Haematol *76*: 230-236, 2006.


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