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Analysis of K-Ras Oncogene Codon 12 Mutations in a Series of Human Lung Cancers

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Abstract: Lung cancer is the major cancer in Turkey; 45% of the deaths due to cancer in Turkish males are from lung cancer. There are 8 histopathological types of lung cancer, and most types have several genomic/genetic alterations, including amplification of oncogenes. The current study evaluated the ras oncogene in human lung cancers from Turkish patients. Thirty-six normal and pathological lung tissue specimens were obtained from patients with chest disease during surgery, frozen at -86°C, and processed within 2-3 months. Mutations in K-ras, codon 12 were analyzed using a series of DNA purification proceedures, polymerase chain reaction amplification, and dot-blotting techniques. Fifteen of the 21 cancer tissues had K-ras mutations. None of the 11 normal lung tissues nor any of the 4 other pathological tissues showed K-ras mutations. Seven of 11 epidermoid carcinomas, 3 of 3 adenocarcinomas, 2 of 3 bronchial carcinomas, 1 of 2 mesotheliomas, 1 of 1 metastatic anaplastic carcinoma, and 1 of 1 rabdomyosarcoma showed point mutations in codon 12, base pairs n 34 and/or 35. Base pair n 34 was mutated G->A in 11 samples and G->T in 4 samples, while base pair n 35 was mutated G->T in 8 samples. There were 3 triple and 3 double mutations in this group of cancers. Because G->A and G->T mutations are considered to be related to specific chemicals in cigarette smoke, and because 13 of the 15 cancer patients, who had K-ras mutations, were heavy smokers, the results from this study directly support, at the molecular level, close linkages between cigarette smoking and carcinogenic mutations in human lung tissues.

Key Words: K-ras codon 12 mutations, non small cell lung cancers.

Introduction

Lung cancer is a major cause of death in both agricultural and industralized countries. It is the major cancer in both men and women in the USA and in much of Europe. Lung cancers are histopathologically classified as small cell lung cancers (SCLC) (subtypes 1) carcinoid, 2) atypical/varient, and 3) classical) and non small cell lung cancers (NSCLC) (subtypes 4) squamous cell carcinoma, 5) epidermoid carcinoma, 6) adenocarcinoma, 7) large cell carcinoma, and 8) mesothelioma) (1-2). While the major types of lung cancer found in cigarette smokers include all of the SCLCs, as well as the epidermoid carcinomas and adenocarcinomas from the NSCLC group(1-3).

Many genetic and epigenetic changes have been observed in each of these general types of lung cancer.

Both increased oncogene and decreased cancer suppressor gene activities have been reported (3-16). There are mutations/amplifications in the ras, myc, erb-B, src, and fos-jun oncogene families, while mutations/losses in the suppressor genes p53, Rb, WT1, and RCC have been observed. However, with 7-8 types/subtypes of lung cancer and more than 15 possible gene/subgene changes, it is not yet understood which gene/subgene plays a critical role in which type/subtype of lung cancer.

Members of the ras family of oncogenes are frequently mutated/amplified in NSCLC, but not SCLC (5,10,17-20). The ras family includes H-ras, K-ras, and N-ras genes located on chromosomes 11, 12, and 1, respectively. The base pair sequences for the exons of each ras are >80% homogeneous; therefore the protein products are similar in tertiary structure and biological

function. These ras proteins are part of the GTP binding protein family, and similar to the alpha subunit of G-protein. They act as an intracellular signal transduction mechanism (17-20).

The normal, non-mutated ras protein is in low quantity in most normal cells, and has a normal alpha subunit of G protein-like kinetic characteristics. However, a single base pair mutation or base substitution in codons 12, 13, or 61 cause the subsequently mutated ras protein to be in an 'active' state. Such a mutant ras protein, together with an amplification of one of the myc oncogenes/oncoproteins, can cause an agressive cancer. In the current study, we evaluated the mutation rate of codon 12 in the K-ras gene in human NSCLC.

Materials and Methods

Surgical tissue specimens were obtained from the Ankara Ataturk Chest Diseases and Chest Surgery Hospital, Chest Surgery Services. Lung cancer, nearby lung tissues and normal lung tissues were taken during open chest surgery. Thirty-six normal and cancer tissue specimens were obtained during a 6-month period and frozen at -86oC until processed within 2-3 months. Pathology was performed by the Pathology Diagnostic Unit of the above mentioned hospital. An analysis of K-ras (codon 12) mutations in 11 normal lung tissues, 21 cancer tissues and 4 other diseased lung tissues are reported here.

Preparation of nucleic acids and the purification of the DNA were accomplished by standard procedures (21,22). In brief, tissues were homogenized in 10 mM Tris-HCl buffer at pH 8.0 containing 0.25 mM EDTA, 100mM NaCl, 0.5% SDS, and 0.25 mg/ml of Proteinase-K. Subsequent purification included the following extractions: 1) 5mg/ml of 8-hydroxy kinolin and saturated phenol in 50mM Tris-HCl buffer at pH 10.5, 2) chloroform:isoamyalcohol (24:1), 3) 3M sodium acetate at pH 5.5, 4) and cold 100% ethanol. Extraction temperatures, times, and subsequent centrifugations are described in ref 23-24. The final extract of purified DNA was concentrated using a 'Speed-Vac' concentrator.

Purified DNA was analyzed at 230, 260, 280, and 325nm and incubated with RNAse to determine DNA quantity and quality. Samples were electrophoresised on standard agarose gels of 0.7, 1.2, 2.4, 4, and 6% to evaluate the molecular weight spectra. DNA marker 1 was bacteriophage lamba cl8571 Sam 7 cleaved with Hind III restriction endonuclease, and DNA marker 2 was DNA pUC19 cleaved with Hinf I restriction endonuclease. Our purified DNA was >99% DNA and showed a range of molecular weights from 23000 to 75 base pairs.

The polymerase chain reaction procedure utilized 2 primers both focusing upon the K-ras gene, number 12 codon (base pairs no 34, 35, 36; 5' to 3'). Primer 1 was 5'-ATG-ACT-GAA-TAT-AAA-CTT-GTG-GTA,3'. Primer 2 was: 5'-G-ACT-GAA-TAT-AAA-CTT-GTG-GTA-GTT-GGA-3'. The normal codon/base pair sequence for the first 12 codons of the K-ras oncogene are 5'ATG-ACT-GAA-TAT-AAA-CTT-GTG-GTA-GTT-GGA-GCT-GGT--3'. The reaction was carried out using a PCR amplification kit for the ras oncogene from Epicenter Corp, USA. The incubaton times were 96°C for 5 min, and then 30 cycles of 56°C, 74°C, and 96°C for 1 min each. Products of these incubations were analyzed by 1, 2 and 6% agrose gel electrophoresis (21-25).

PCR amplified normal and cancer tissue DNA were subsequently analyzed by the dot-blot method (23-24) using a 40 well dot-blot manifold (Biorad). 100 μg of each DNA was incubated with the following biotin labeled hybridization solutions: normal codon -5'GGT3', mutant n 1 - 5'TGT3', mutant n 2 - 5'CGT3', mutant n 3 - 5'AGT3', mutant n 4 - 5'GTT3', mutant n 5 - 5'GAT3', and mutant n 6 - 5'GCT3'. Subsequently, streptavidin-alkaline phosphatase, and BICP were added. Buffer concentrations, incubation times and temperatures, and technical procedures are described in detail in references 23 and 24.

Results

Figure 1 shows a typical 2% agrose gel electrophoresis analysis of the PCR products when primer 1 was used. The major nucleotide product was a 75 base pair product, which was observed in all samples.

PCR was performed on the DNA purified from 36 normal and pathological lung tissues. The PCR products were analyzed for mutations in the K-ras oncogene, codon 12, base pairs n 34 and 35. Fifteen of 21 cancer tissues had K-ras mutations: 7 of 11 epidermoid carcinomas, 3 of 3 adenocarcinomas, 2 of 3 bronchial carcinomas, 1 of 2 mesotheliomas, 1 of 1 metastatic amaplastic carcinoma, and 1 of 1 rhabdomyosarcoma (Table 1). We observed no mutations in codon 12 in any of the 11 normal lung tissues nor in any of the 4 other diseased lung tissues (data not given).

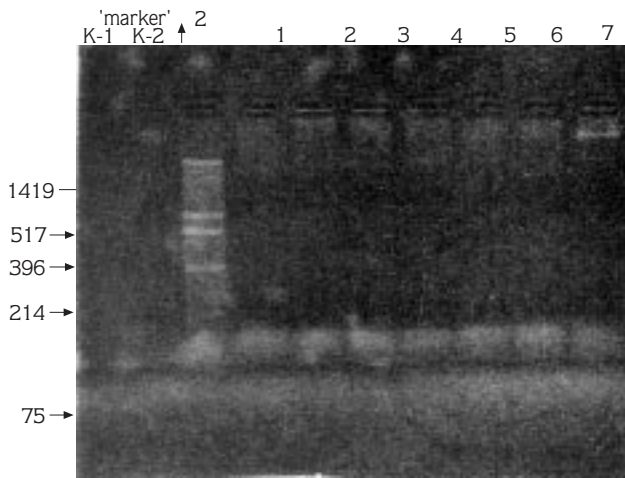A typical dot-blot hybridization analysis for mutant n 3 of K-ras, codon 12 (5'AGT3') is given in Figure 2.

No Pathology	Grade	Mutation	Occupation	Yrs Smoking
1 Meta Anapl Carcinoma	2	M3	Farmer	0
2 Epidermoid	5	M3, M5	Indust Worker	43
3 Epidermoid	4	M3, M4	Farmer	35
4 Adenocarcin	4	M4	Indust Worke	40
5 Bron Carcin	3	M3	Constr Worker	0
6 Epidermoid	4	M3	Driver	40
7 Adenocarcin	2	M3, M4	Businessman	50
8 Epidermoid	3	M3	Tailor	24
9 Epidermoid	4	M1, M3, M4	Farmer	25
10 Adenocarcin	4	M3, M4	Driver	20
11 Rabdomyosarc	3	M4	Indust Worker	20
12 Bron Carcin	2	M1	Constr Worker	52
13 Epidermoid	4	M1, M3, M4	Indust Worker	45
14 Mezothelioma	3	M1	Constr Worker	0
15 Epidermoid	3	M3, M4	Industr Worker	43

Mutant n 3 was observed in cancer tissues from patients n 1, 2, 3, 5, 6, 7, 8, 9, 10, 13, and 15, but not in other patients's cancer tissues or in any normal lung tissues (also summarized in Table 2).

A general summary of the analysis of lung cancer K-ras, codon 12 mutations are given in Table 1. All of the 15 patients were males. Fourteen patients were blue-collar, workers, while twelve patients were heavy cigarette smokers. NSCLC of the epidermoid and adenocarcinoma subtypes were the most commonly observed cancers. Bronchial carcinoma, mesothelioma, rhabdomyosarcoma, and metastatic anaplastic carcinoma were also identified and reported from the 6 month collection analysis.

Table 2 summarizes the analysis of lung cancer K-ras, codon 12, base pair mutations. Base pair n 34 was most often mutated with a guanine to adenine transition (11 times) and guanine to thymine transversion (4 times). The second most observed mutation was in base pair n 35, which showed a guanine to thymine transversion (8 times). The other 3 mutation possibilities in codon 12, base pairs n 34 and/or n 35 were not observed or found only 1 time. Codon 12, base pair n 36 was not studied. From the 7 epidermoid carcinomas, we observed 3 triple and 3 double mutations, while 2 of the 3 adenocarcinomas showed double mutations. Normal lung tissue taken from nearby or distal areas (from tumor) were not found to contain any K-ras gene, codon 12 mutations.

Table 2.   Summary of K-Ras Codon 12 Mutations in Lung Cancer.

A) Pathology	M1 5'TGT3'	M2 5'CGT3'	M3 5'AGT3'	M4 5'GTT3'	M5 5'GAT3'	M6 5'GCT
Epidermoid	9,13	–	2,3,6,8 9,13,15	3,9,13 15	2	–
Adenocarcin	–	–	7,10	4,7,10	–	–
Bron Carcin	12	–	5	–	–	–
Mesothelioma	14	–	–	–	–	–
Rabdomyosarc	–	–	–	11	–	–
Meta Anapl Carcinom	–	–	1	–	–	–
Totals	4	0	11	8	2	0

B)	codon 12, base pair n 34	codon 12, base pair n 35
	G to T = 4	G to T = 8
	G to C = 0	G to A = 1
	G to A = 11	G to C = 0

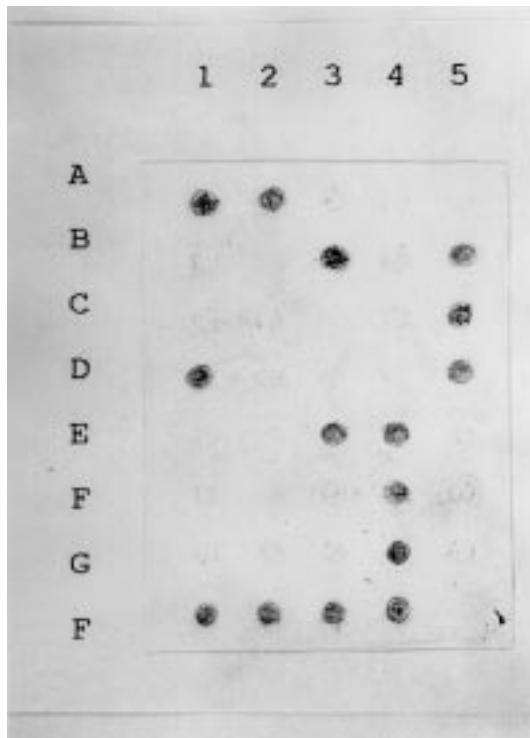– codon 12, base pair n 36 was not analyzed.

Figure 2.   Dot-blot hybridization analysis for mutant no 3 of K-ras, codon 12 (5'AGT3'). Well numbers and patient numbers are as follows: A1-1, A2-2, B3-3, B4-4, B6-5, C5-6, D1-7, D5-8, E3-9, E4-10, F1-11, F2-12, F4-13, G3-14, and G4-15. H1, H2, H3, and H4 wells were positive controls. All other wells contained PCR products from normal lung tissues, nearby lung tissues, and cysts; they did not show any mutations.

Discussion

Genetic changes in most cancers, including lung cancers, are considered to be a result of a multistep process of pathogenesis. Such changes in NSCLC involve mutations, substitutions, and deletions of base pairs or DNA segments of the ras oncogene family (3-5,25-33). Single base pair substitutions in H-ras, K-ras, and N-ras in codons 12, 13, and/or 61 can lead to ras proteins which are defective in their GTPase activities. These 'mutated' ras proteins then remain in an 'active' state and stimulate a group of protein kinases, which provide the cancer cell with a switched on mechanism that signals rapid cell proliferation (11,18,19).

A point mutation in the K-ras codon was one of the first mutations observed in cancer cells (17,25) and is currently one of the most promising targets for gene therapy (30-39). Among the NSCLC subtypes, K-ras, codon 12 has been reported to be mutated from 15 to 60% in squamous cell carcinomas and 40 to 65% in adenocarcinomas (10,11,13). Mutations within each of the codons 12, 13, and 61 have been reported in NSCLC; however, we only examined exon 12 in the current study. We found K-ras, codon 12 to be mutated 77% in epidermoid carcinoma, 100% in adenocarcinoma, and 66% in bronchial carcinoma. The most common single base pair substitutions within codon 12 have been reported to be G to T and G to A changes (25,26,30-32). We observed mutations of G to T in base pair n 34 at 16%, G to T in base pair n 35 at 33%, G to A in base pair

n 34 at 46%, and in all other base pair changes at less than 5%. Therefore, 95% of the base pair mutations in our current studies were G to T and/or G to A transversions.

Literature reports do not support any correlations between K-ras mutations and cancer metastases (33). Our data also do not show relationships between K-ras and metastases. We found no correlations between K-ras mutations and pathology grade.

Base pair mutations of the G to T type and G to A type may relate to the action of two types of chemical carcinogens which are high in cigarette smoke, benzo(a)pyrene and the N-nitroso compounds (28,32). Within the cancer tissues of our NSCLC patients had been smoking for more than 20 years, the K-ras, codon 12 analysis showed that 100% had the G to T mutations (base pairs no 34 and 35) and/or 77% had the G to A mutations (base pairs no 34 and 35). Our data supports the hypothesis that specific genomic mutations, even single base pair mutations, may be correlated with specific groups of chemical carcinogens.

In addition, NSCLC adenocarcinomas with K-ras mutations have been correlated with decreased patient survival time. This was especially true in early stage cancers (4,5,28,33). We are monitoring the patients in the current study to determine prognosis. Specific K-ras mutations may thus help identify a population of lung cancer patients which have a poor prognosis. If this can be varified for each NSCLC subtype, along with perhaps other specific genomic alterations, such data should help the clinician in determining the extent of classical multi-modal therapies, and in selecting more specific new therapies such as gene therapies (34-39).

Acknowledgements

References

1. Minna JD, Seikdo Y, Fong KM, Gazdar AF. Cancer of the Lung. Cancer:Principles and Practices of Oncology (Eds. VT DeVita, S Hellman and SA Rosenberg) Lippincott, Philadelphia 1997, pp 849-943.

2. Beahrs H, Henson DE, Hutter RVP, Kennedy BF. Lung. Manuel for Staging of Cancer (American Joint Commitee on Cancer, Chicago USA) edition no 4, 1992, pp:115-122.

3. Minna JD. The molecular biology of lung cancer pathogenesis. Chest 103:449S-456S, 1993.

4. Gazdar AF. Molecular markers for the diagnosis and prognosis of lung cancer. Cancer 69: 1592-1599, 1992.

5. Miyamoto H, Harada M, Isobe H, Dosaka-Akita H, Haneda H, Yam-aguchi E. Prognostic value of nuclear DNA content and expres-sion of ras oncogene in lung cancer. Cancer Res 51: 6346-6350, 1991.

6. Koo HM, Monks A, Mikheev A, Rubenstein V, Vande Wouds GF. Enhanced sensitivity to arabinofuranosylcytosine and topoi-somerase II inhibitors in tumor cell lines harboring activated ras oncogenes. Cancer Res 56:5211-5216, 1996.

7. Xu J, Gimenez-Conti I, Cunningham JE, Collet AM, Luna MA, Lanfranchi HA, Spitz MR, Conti CJ. Alterations of p53, cyclin D1, Rb, and H-ras in human carcinomas related to tobacco use. Cancer 83:204-212, 1998.

8. DePinho RA, Schreiber-Agus N, Alt FW. Myc family oncogenes in the development of normal and neoplastic cells. Adv Cancer Res 57:1-46, 1991.

9. Prins J, DeVries EGE, Mulder NH. The myc family of oncogenes and their presence and importance in small cell lung carcinoma and other tumors. Anti-Cancer Res 13:1373-1386, 1993.

10. Volm M, Drings P, Wodrich W, van Kaick G. Expression of onco-proteins in primary human non small cell lung cancers. Clin Exp Metastasis 11: 325-329, 1993.

11. Sabichi AL, Birrer MJ. Regulation of nuclear oncogenes expressed in lung cancer cell lines. J Cell Biochem Suppl 24: 218-227, 1996.

12. Volm M. P-Glycoprotein associated expression of c-fos and c-jun protein products in human lung carcinomas. Anti-Cancer Res 13:375-378, 1993.

13. Schauer I, Siriwardena S, Langan TA, Sclafani RA. Cyclin D1 overexpression vs Rb inactivation, implications for growth control evasion in non-small cell and small cell lung cancers Proc Natl Acad Sci, USA 91:7827-7831, 1994.

14. Volm M, Efferth T, Mattern J. Oncoproteins (c-myc, c-erb-B1, c-erb-B2, c-fos) and suppressor gene products (p53) expression in squamous cell carcinomas of the lung: clinical and biological correlations. Anti-Cancer Res 12: 11-20, 1992.

15. Reissman PT, Koga H, Takahashi R, Figlin RA, Holmes EC, Piantadosi S, Cordon-Cardo C, Salamon DF. Inactivations of Rb susceptibility gene in non small cell lung carcinoma. Oncogene 8: 1913-1919, 1993.

16. Dosaka-Akita H, Hu S-X, Fujino M, Harada M, Benedict WF. Altered retinoblastoma protein expression in non small cell lung cancer. Cancer 79: 1329-1337, 1997.

17. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res 49: 4682-4689, 1989.

18. Egan SEW, Weinberg RE. The pathway to signal achievement. Nature (London) 361: 781-783, 1993.

19. Marais R, Light Y, Paterson HF, Marshall CJ. Ras recruits raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J 14: 3136-3145, 1995.

20. Kodaki T, Woscholski R, Hallberg B, Rodriquez-Viciana P, Downward J, Parker PJ. Activation of phosphatidylinositol 3 kinase by ras. Curr Biol 4:798-806, 1994.

21. TÜBITAK, Beta talaseminin moleküler hibridizasyon, Yöntemleri ile tanysy ve incelemesi, 1991.

22. Ausubel AM. Current Protocols in Molecular Biology, John Wiley and Sons, NY, 1987, chapter 2.

23. Vries V, Bogard MA, Eist H, Boom JH, Ed A, Bos LJ. A dot-blot screening procedure for mutated ras oncogenes using synthetic oligonucleotides. Gene 50: 313-320, 1986.

24. Bayer EA, Ben-Hur H, Wilchek M. Avidin-biotin technology. Meth in Enzymology 184: 425-426, 1990.

25. Lowry DR. Function and regulation of ras oncogene. Ann Rev Biochem 62: 851-891, 1993.

26. Rodenius S, Van de Wetering M, Walter JM, Evers SG. Mutational activation of K-ras oncogene: a possible pathologetic factor in adenocarcinomas of the lung. New Eng J Med 15: 929-936, 1987.

27. Krontikiris TG, Devlin B, Karp DD, Roberts NJ, Risch N. An association between the risk of cancer and mutations in the HRAS-1 mimisatellite locus. New Eng J Med 329: 517-523, 1993.

28. Mitsudomi T, Viallet J, Mulshine JL, Linnvila RI, Minna JD, Gazdar AF. Ras gene mutations in non small cell lung cancers are associated with shortened survival irrespective of treatment intent. Cancer Res 51: 4999-5002, 1991.

29. Suzuki Y, Orita M, Shiraishi M, Hayashi K, Sekiya T. Detection of ras gene mutations in human lung cancers by single strand conformation polymorphism analysis. Oncogene 5: 1037-1043, 1990.

30. Mitsudomi T, Viallet J, Mulshine JL, Iliona R. Mutations of ras genes distinguish a subset of NSCLS cell lines from SCLC cell lines. Oncogene 6: 1353-1362, 1991.

31. Capon DJ, Seeburg PH, McGrath JP,Hayflick JS, Edman U. Activation of Ki-ras 2 gene in human colon and lung carcinomas by two different point mutations. Nature 304: 507-514, 1990.

32. Reynold SH, Anna KC, Brown KC, Weist JS. Activated proto-oncogenes in human tumors in smokers. Proc Natl Acad Sci, USA 88: 1085-1089, 1991.

33. Rossel RLS, Skacel Z, Mates JL, Maestre J. Prognostic impact of mutated K-ras gene in surgically resected NSCLC. Oncogene 8: 2407-2412, 1993.

34. Anderson F. Gene therapy. Sci American 27: 124-128, 1995.

35. Gibson I, Anti-sense approaches to gene therapy of cancer. Cancer and Metastasis Rev 15: 287-299, 1996.

36. Crooke ST, Bennett CF. Progress in anti-sense oligonucleotide therapeutics. Ann Rev Pharm Toxicol 36: 107-129, 1996.

37. Monia BP, Johnston JF, Ecker DJ, Zounes MA, Lima WF, Freier SM. Selective inhibition of mutant H-ras mRNA expression by antisense oligonucleotides. J Biol Chem 267: 19954-19959, 1992.

38. Gray GD, Hernandez OM, Hebel D, Root M, Pow-Sang JM, Wickstrom E. Antisense DNA inhibition of tumor growth induced c-Ha-ras oncogene in nude mice. Cancer Res 53: 577-581, 1993.

39. Chen G, Oh S, Monia BP, Stacey DW. Antisense oligonucleotides demonstrate dominant role of c-Ki-ras proteins in regulating the proliferation of diploid human fibroblasts. J Biol Chem 271: 28259-28263, 1996.