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Frequent Allelic Deletion at a Novel Locus on Chromosome 5 in Human Lung Cancer[1]

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ABSTRACT

Frequent allelic deletions in tumor cells are indicative of the inactivation of tumor suppressor genes. Recently, we isolated the single-copy sequence del-27 (I. Wieland, M. Böhm, and S. Bogatz, Proc. Natl. Acad. Sci. USA, *89:* 9705–9709, 1992). Here we show that del-27 detects a restriction fragment length polymorphism that allows examination for loss of heterozygosity (LOH) in tumor specimens. LOH at the *del-27* locus occurred in 57% (4 of 7) of the informative lung carcinomas independent of the histopathological differentiation grade. LOH for exon 11 of the *APC* gene occurred in 71% (5 of 7) of the informative cases but was not associated with LOH at the *del-27* locus. The del-27 sequence was localized to chromosome 5p13–5q14, proximal to the MCC/APC region, using a somatic cell hybrid panel. Together with our previous finding that del-27 is deleted homozygously in a lung carcinoma cell line, these results suggest that del-27 is linked closely to a novel putative tumor suppressor gene.

INTRODUCTION

Genetic alterations on chromosome 5 have been demonstrated in a variety of human neoplasias. Loss of an entire chromosome 5 or interstitial deletions in the long arm (5q−) are observed frequently in patients with acute myelogenous leukemia or myelodysplastic syndrome (1). These are probably mutagen-induced leukemias occurring as a late complication of cytotoxic therapy or as a result of occupational exposure to carcinogens (2). The smallest commonly deleted region in these neoplastic diseases has been narrowed to 5q31. This region contains the *IRF-1* (interferon regulatory factor 1) gene and possibly another putative tumor suppressor gene (2, 3). In some solid tumors, allelic deletions of the *MCC* and *APC* tumor suppressor genes have been reported at 5q21. The *MCC* and *APC* genes originally were discovered as potential tumor suppressor genes of sporadic and hereditary forms of colorectal carcinomas (4, 5). Also, a high incidence of allelic deletions in the MCC/APC region is found in esophageal and small cell lung carcinoma, both correlated with excessive mutagen exposure (6, 7). From cytogenetic studies on lung carcinomas it appears that probably a larger chromosomal region (5q13–5q21) is involved in lung tumorigenesis (8).

To identify DNA sequences that are specifically deleted in tumor cells, we recently isolated a DNA probe (del-27) from human chromosome 5 by using genomic difference cloning (9, 10). This sequence is deleted homozygously in a lung carcinoma cell line, suggesting its linkage to a putative tumor suppressor gene (10). Here we show that the del-27 sequence is located in chromosomal region 5p13–5q14 and detects a RFLP.[4] Using the del-27 sequence as a RFLP probe, we observe allelic deletion in 57% of the squamous cell carcinomas of the lung.

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[4] The abbreviations used are: RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; LOH, loss of heterozygosity; NSCLC, non-small cell lung carcinoma.

MATERIALS AND METHODS

PCR on Human/Hamster Somatic Cell Hybrids. Human/hamster somatic cell hybrid cell lines GM 10114, GM 11443, GM 11444, GM 11437, GM 11442, and GM 11436 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). High molecular weight DNA was isolated (11) and 100–200 ng were used as template DNA in PCRs. Amplifications were carried out in a 50-μl reaction volume containing 20 pmol of each del-27 primer (5′GGATAACAAAAGGGATGTGC3′ and 5′GATTGCATCAGCTGCACCC3′) (10), 200 μM concentrations of deoxynucleotide triphosphates, and 1 unit of Taq DNA polymerase (Boehringer Mannheim) at 95°C for 5 min followed by 35 cycles at 94°C for 1 min, 58°C for 2 min, and 72°C for 1.5 min. PCR products were analyzed on 2% agarose gels.

RFLP Analysis by Southern Blot Hybridization and PCR. High molecular weight DNA (11) from randomly chosen normal blood donors was cleaved to completion with 14 different restriction enzymes in independent reactions. The digests (5 μg) were electrophoresed on 0.8% agarose gels and transferred onto a nylon membrane (Gene Screen Plus; Du Pont) using a vacuum blotting unit (Vacugene; LKB). The blots were hybridized to a ^{32}P-labeled del-27 probe (9) for 18 h at 64°C and washed three times in 2 × standard saline-citrate-0.2% sodium dodecyl sulfate (11) and once in 0.5 × standard saline-citrate-0.2% sodium dodecyl sulfate at 64°C. Autoradiography was performed as described (9).

RFLP analysis at the *APC* locus (a *Rsa*I polymorphic site in exon 11 of the *APC* gene) was performed by PCR on isolated DNA from blood donors as described (12). The generated 133-base pair PCR product is cleaved (allele *b*) to an 85- and a 45-base pair fragment by *Rsa*I digestion if the polymorphic restriction site is present, and it remains uncleaved (allele *a*) if the site is absent (12). *Rsa*I-digested PCR products were electrophoresed on 3% agarose gels.

Cell Lines and Tumor Specimens. Human lung cancer cell lines SK-LU-1 (HTB 57), SW 900 (HTB 59), and NCI-H69 (HTB 119) were obtained from the American Type Culture Collection; all other cell lines were obtained and cultivated as described (10). Matching normal blood and tumor biopsies from lung cancer patients were collected as described (13). To determine allelic deletions, tumor tissue was purified for Southern blotting by microscopy-guided gross dissection (14) and for PCR analysis by a microdissection method (13). Southern blotting, hybridizations, and PCRs were carried out as described above.

RESULTS

Subchromosomal Localization of the del-27 Sequence. Recently, the del-27 sequence was assigned to human chromosome 5 using a human/hamster somatic cell hybrid panel (10). To localize the del-27 sequence more closely, human/hamster somatic cell hybrids retaining only parts of human chromosome 5 were analyzed by PCR. In all hybrids having deletions of the short arm of human chromosome 5 (GM 11437, GM 11442, and GM 11436), a 356-base pair PCR product of the del-27 sequence was amplified (Fig. 1). In the hybrid GM 11443, containing human chromosome 5 with an interstitial deletion [del(5)(q15q21.3)] which encompasses the MCC/APC region (data not shown), the del-27 PCR product also was detected. However, in hybrid GM 11444, retaining only the distal part of 5q (5q22–ter), no del-27 PCR product was amplifiable (Fig. 1). The same results were obtained by genomic Southern blotting (data not shown). This most likely places del-27 proximal to the MCC/APC region. From these results we conclude that the del-27 sequence maps to human chromosome 5p13–5q14.

RFLP Analysis at the *del-27* Locus. A RFLP was detectable in *Hind*III-cleaved DNA from randomly chosen normal blood donors. Seven of 13 donors (54%) were heterozygous at the *del-27* locus with

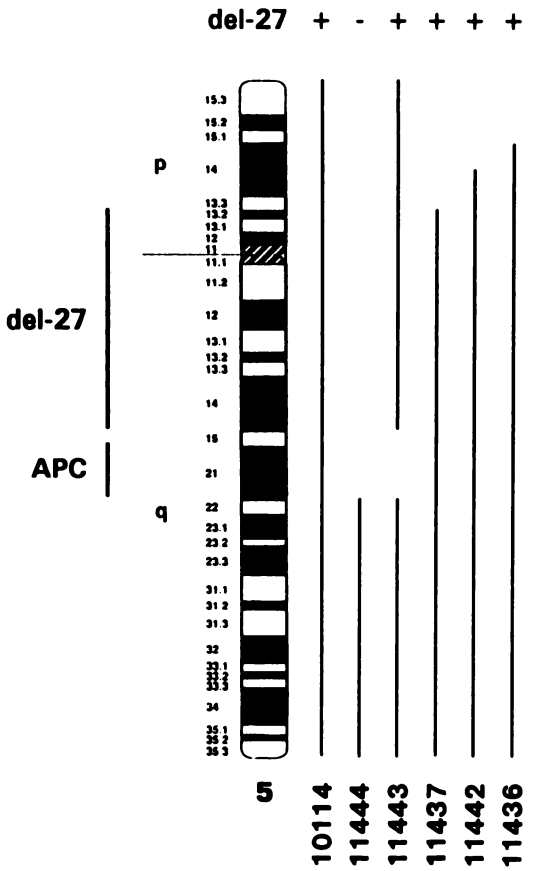

Fig. 1. Subchromosomal localization of the del-27 sequence on human chromosome 5 by PCR. Diagram depicts human/hamster somatic cell hybrid cell lines retaining different parts of human chromosome 5. +, presence; –, absence of the del-27 PCR product.

frequencies for the 4.8-kilobase fragment (allele *a*) of M = 0.27 and for the 3.9-kilobase fragment (allele *b*) of M = 0.73. These donors showed a similar frequency of heterozygosity for the APC *Rsa*I polymorphism (54%). However, occurrence of the del-27 and APC polymorphisms was distinct. This was most apparent in human lung carcinoma cell lines. Although 7 of 8 (88%) NSCLC cell lines tested were homozygous at the *del-27* polymorphic site, 4 of the 8 (50%) NSCLC cell lines were heterozygous at the *APC* locus (Fig. 2, *Lanes b–h* and *m*). In the small cell lung carcinoma cell line SK-LC-17, which has a homozygous deletion of the del-27 sequence (10), allele *a* of the APC RFLP is still contained (Fig. 2, *Lane i*). Since no matching normal DNA of these lung cancer cell lines was available, allelic deletion at the *del-27* locus was investigated in tumor biopsies.

Allelic Deletion at the *del-27* Locus in Human Squamous Cell Carcinomas. Of 14 patients with squamous cell lung carcinoma, 7 were heterozygous (*i.e.*, informative) for the *del-27* polymorphic site. In 4 of these 7 informative cases (57%) LOH was observed in the carcinoma (Fig. 3 *A*; Table 1). This is a high frequency of allelic loss compared with other loci (15–17) and considering the small sample size. These four carcinomas consisted of one well, two moderately, and one poorly differentiated squamous cell lung carcinoma (18), which does not suggest a correlation between allelic loss at the *del-27* locus and differentiation grade. We also looked for allelic loss at the *APC* locus at 5q21 by PCR. To exclude contaminating normal stromal cells and leukocytes, which were responsible for visible background bands in Southern blot hybridizations (Fig. 3*A*, *P11, P19, P56*) and might have obscured PCR results, tumor islets free of contaminating normal cells and necrotic tissue were isolated by a microdissection method (13). With this procedure, a high frequency (71%) of loss of heterozygosity also was observed for the *APC* gene (Fig. 3; Table 1). Two of the four squamous cell carcinomas that showed allelic deletion at the *del-27* locus also showed allelic deletion at the the *APC* locus (Table 1). This demonstrates that a high frequency of allelic loss can occur at either *del-27*, *APC*, or at both loci on chromosome 5 in human squamous cell carcinoma of the lung.

DISCUSSION

The main result of this study is the identification of another region on human chromosome 5 (5p13–5q14) that shows frequent allelic

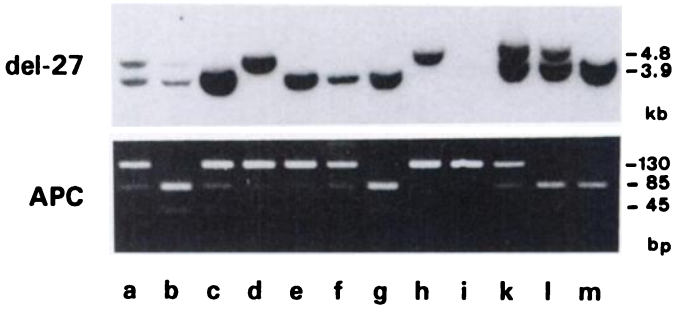

Fig. 2. RFLP analysis at the *del-27* and *APC* loci in lung cancer cell lines. Human peripheral blood leukocytes (*Lane a*), human NSCLC cell lines Calu-3 (*lane b*), A-427 (*Lane c*), A-549 (*Lane d*), LXF289 (*Lane e*), SK-LC-12 (*Lane f*), LX-1 (*Lane g*), SK-LU-1 (*Lane h*), SW 900 (*Lane m*), and small cell lung carcinoma cell lines SK-LC-17 (*Lane i*), NCI-H69 (*Lane k*), and NCI-H417 (*Lane l*) were analyzed by *Hin*dIII cleavage and Southern blot hybridization with the del-27 probe (*top*) and by APC-PCR and *Rsa*I cleavage (*bottom*). *Right ordinate*, band sizes. *kb*, kilobases; *bp*, base pairs.

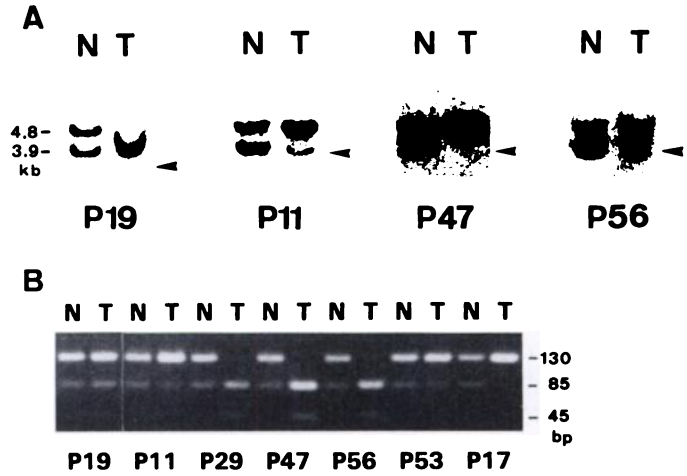

Fig. 3. Allelic deletion of the del-27 sequence and in the *APC* gene in tumor biopsies from lung cancer patients. (*A*) Southern blots of *Hin*dIII-cleaved DNA from matching normal peripheral blood leukocytes (*Lanes N*) and tumor tissue (*Lanes T*) from four patients with squamous cell lung carcinoma (*P19, P11, P47*, and *P56*) hybridized with the del-27 RFLP probe. *Arrowheads*, loss of heterozygosity in the tumor. (*B*) PCR analysis of the *APC* gene exon 11 for loss of heterozygosity in tumor cells (*Lanes T*) isolated from patients with squamous cell lung carcinoma (*P19, P11, P29, P47, P56, P53*, and *P17*). *Lanes N*, PCR results of matching normal cells. *Ordinate*, band sizes. *kb*, kilobases; *bp*, base pairs.

Table 1 *Allelic deletion at the del-27 and APC loci in human squamous cell lung carcinomas*

Patient	Histology	*del-27*	*APC* exon 11
19	G1[a]	LOH[b]	–
11	G2	LOH	–
24	G2	–	n.d.
29	G2	–	LOH
46	G2	–	n.i.
53	G2	n.i.	LOH
56	G2	LOH	LOH
17	G3	n.i.	LOH
47	G3	LOH	LOH
Allelic loss		57% (4/7)	71% (5/7)

[a] Differentiation grade: well (G1); moderately (G2); or poorly (G3) differentiated according to World Health Organization guidelines.
[b] LOH, loss of heterozygosity (*i.e.*, allelic loss observed); –, no allelic loss observed; n.i., not informative; n.d., not determined.

deletion in squamous cell lung carcinomas. Loss of heterozygosity in this region was detected with the del-27 sequence, which also is deleted homozygously in a human lung carcinoma cell line. This supports the close linkage of the del-27 sequence to a novel putative tumor suppressor gene involved in the development of lung cancer.

Previous studies have demonstrated that recurrent allelic deletions in tumors are indicative of the inactivation of a tumor suppressor gene. These tumor-specific allelic deletions can be detected by RFLP probes that show LOH in the tumor cells. Apparently, the closer a RFLP probe is to the relevant tumor suppressor gene, the higher is the incidence of LOH (16). In human lung cancer, LOH frequently has been observed in the region of the *p53* gene (17p13), the MCC/APC region (5q21), and at least 3 regions on the short arm of chromosome 3 (7, 19). In addition to allelic deletions, homozygous deletions are considered evidence for the identification of tumor suppressor genes (20–22). Homozygous deletions in the *p53* gene and on chromosome 3p were reported in few lung carcinoma cell lines and lung carcinomas, implying the truly recessive way of gene inactivation (13, 23–25).

The del-27 RFLP shows 54% heterozygosity in normal DNA. A similar frequency of heterozygosity was observed in exon 11 of the *APC* gene in normal DNA (12) (Fig. 2). Heterozygosity rates well over 50% have been reported for other tumor suppressor loci (12). However, most (7 of 8) NSCLC cell lines were homozygous at the *del-27* locus, whereas only one-half of these tumor cell lines were homozygous at the *APC* locus. Although no matching normal tissue was available for these tumor cell lines it is reasonable to assume the heterozygosity rate of normal DNA; *i.e.*, that approximately one-half of the matching normal tissue were heterozygous. This suggests that LOH at the *del-27* locus plays a major role in NSCLC cell lines in contrast to LOH at the *APC* locus. In fact, several lung cancer cell lines express the normal APC protein (26).

In human squamous cell lung carcinomas, LOH was 71% at the *APC* locus (Table 1). In previous reports on NSCLC, only 40% LOH was observed for the *APC* gene and 20–30% LOH was detected (with the L5.71 probe) for the *MCC* gene (7, 16, 17). This discrepancy may be a result of different purity grades of the examined tumor tissue. A high incidence of allelic deletion for the *APC* gene was observed in esophageal cancer (66%) and in small cell lung carcinoma (70%) (7, 15). Recently, it has been shown that the APC protein associates with β-catenin (27, 28), which functions as a cytoplasmic anchor protein of the cadherin cell-cell adhesion molecules. Loss or disturbance of E-cadherin-mediated adhesion correlates with dedifferentiation and metastasis of squamous cell carcinomas of the lung (18). Taken together these results suggest loss of APC and cell-cell adhesion in the later stages of lung tumorigenesis (for review, see Ref. 29).

In the same specimens from human squamous cell carcinomas of the lung, LOH at the *del-27* locus was 57%; it occurred independent from LOH at the *APC* locus, and it also appeared independent from the differentiation grade of the seven examined carcinomas. This indicates that the *del-27* and *APC* loci are not closely linked. Furthermore, the *del-27* locus was mapped proximal (5p13–5q14) to the MCC/APC region (5q21). A more precise localization of *del-27* should be possible by fluorescence *in situ* hybidization. In conclusion these results show that *del-27* is a novel chromosome 5 locus implicated in lung tumorigenesis.

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REFERENCES

1. Fourth International Workshop on Chromosomes in Leukemia 1982. Deletion of 5q. Cancer Genet. Cytogenet., *11:* 296–299, 1984.
2. Le Beau, M. M., Espinosa III, R., Neuman, W. L., Stock, W., Roulston, D., Larson, R. A., Keinanen, M., and Westbrook, C. A. Cytogenetic and molecular delineation of the smallest commonly deleted region of chromosome 5 in malignant myeloid diseases. Proc. Natl. Acad. Sci. USA, *90:* 5484–5488, 1993.
3. Willman, C. L., Sever, C. E., Pallavicini, M. G., Harada, H., Tanaka, N., Slovak, M. L., Yamamoto, H., Harada, K., Meeker, T. C., List, A. F., and Taniguchi, T. Deletion of *IRF-1*, mapping to chromosome 5q31.1, in human leukemia and preleukemic myelodysplasia. Science (Washington DC), *259:* 968–971, 1993.
4. Kinzler, K. W., Nilbert, M. C., Vogelstein, B., *et al.* Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. Science (Washington DC), *251:* 1366–1370, 1991.
5. Groden, J., Thliveris, A., Samowitz, W., *et al.* Identification and characterization of the familial adenomatous polyposis coli gene. Cell, *66:* 589–600, 1991.
6. Boynton, R. F., Blount, P. L., Yin, J., *et al.* Loss of heterozygosity involving the *APC* and *MCC* genetic loci occurs in the majority of human esophageal cancers. Proc. Natl. Acad. Sci. USA, *89:* 3385–3388, 1992.
7. D'Amico, D., Carbone, D. P., Johnson, B. E., Meltzer, S. J., and Minna, J. D. Polymorphic sites within the *MCC* and *APC* loci reveal very frequent loss of heterozygosity in human small cell lung cancer. Cancer Res., *52:* 1996–1999, 1992.
8. Miura, I., Graziano, S. L., Cheng, J. Q., Doyle, L. A., and Testa, J. R. Chromosome alterations in human small cell lung cancer: frequent involvement of 5q. Cancer Res., *52:* 1322–1328, 1992.
9. Wieland, I., Bolger, G., Asouline, G., and Wigler, M. A method for difference cloning: gene amplification following subtractive hybridization. Proc. Natl. Acad. Sci. USA, *87:* 2720–2724, 1990.
10. Wieland, I., Böhm, M., and Bogatz, S. Isolation of DNA sequences deleted in lung cancer by genomic difference cloning. Proc. Natl. Acad. Sci. USA, *89:* 9705–9709, 1992.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
12. Greenwald, B. D., Harpaz, N., Yin, J., Huang, Y., Tong, Y., Brown, V. L., McDaniel, T., Newkirk, C., Resau, J. H., and Meltzer, S. J. Loss of heterozygosity affecting the *p53*, *Rb*, and *mcc/apc* tumor suppressor gene loci in dysplastic and cancerous ulcerative colitis. Cancer Res., *52:* 741–745, 1992.
13. Böhm, M., Wieland, I., and Totzeck, B. Detection of tumor-specific homozygous deletions in human biopsies by polymerase chain reaction. Cancer Genet. Cytogenet., *65:* 83–87, 1993.
14. Goelz, S. E., Hamilton, S. R., and Vogelstein, B. Purification of DNA from formaldehyde fixed paraffin embedded human tissue. Biochem. Biophys. Res. Commun., *130:* 118–126, 1985.
15. Huang, Y., Boynton, R. F., Blount, P. L., *et al.* Loss of heterozygosity involves multiple tumor suppressor genes in human esophageal cancer. Cancer Res., *52:* 6525–6530, 1992.
16. Ashton-Rickardt, P. G., Wyllie, A. H., Bird, C. C., Dunlop, M. G., Steel, C. M., Morris, R. G., Piris, J., Romanowski, P., Wood, R., White, R., and Nakamura, Y. *MCC*, a candidate familial polyposis gene in 5q.21, shows frequent allele loss in colorectal and lung cancer. Oncogene, *6:* 1881–1886, 1991.
17. Tsuchiya, E., Nakamura, Y., Weng, S.-Y., Nakagawa, K., Tsuchiya, S., Sugano, H., and Kitagawa, T. Allelotype of non-small cell lung carcinoma: comparison between loss of heterozygosity in squamous cell carcinoma and adenocarcinoma. Cancer Res., *52:* 2478–2481, 1992.
18. Böhm, M., Totzeck, B., Birchmeier, W., and Wieland, I. Differences of E-cadherin expression levels and patterns in primary and metastatic human lung cancer. Clin. Exp. Metastasis, *12:* 55–62, 1994.
19. Minna, J., Maneckjee, R., D'Amico, D., *et al.* Mutations in dominant and recessive oncogenes, and the expression of opioid and nicotine receptors in the pathogenesis of lung cancer. *In:* J. Brugge, T. Curran, E. Harlow, and F. McCormick (eds.), Origins of Human Lung Cancer: A Comprehensive Review, pp. 781–789. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1993.
20. Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature (Lond.), *323:* 643–646, 1986.
21. Gessler, M., Poutska, A., Cavenee, W., Neve, R. L., Orkin, S. H., and Bruns, G. A. P. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature (Lond.), *343:* 774–778, 1990.
22. Fountain, J. W., Karayiorgou, M., Ernstoff, M. S., *et al.* Homozygous deletions within human chromosome band 9p21 in melanoma. Proc. Natl. Acad. Sci. USA, *89:* 10557–10561, 1992.
23. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. Science (Washington DC), *246:* 491–494, 1989.
24. Rabbitts, P., Bergh, J., Douglas, J., Collins, F., and Waters, J. A submicroscopic homozygous deletion at the *D3S3* locus in a cell line isolated from a small cell lung carcinoma. Genes Chromosomes Cancer, *2:* 231–238, 1990.
25. Yamakawa, K., Takahashi, T., Horio, Y., Murata, Y., Takahashi, E., Hibi, K., Yokoyama, S., Ueda, R., and Nakamura, Y. Frequent homozygous deletions in lung cancer cell lines detected by a DNA marker located at 3p21.3–p22. Oncogene, *8:* 327–330, 1993.
26. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Willson, J. K. V., Paraskeva, C., Petersen, G. M., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. The *APC* gene product in normal and tumor cells. Proc. Natl. Acad. Sci. USA, *90:* 2846–2850, 1993.
27. Rubinfeld, B., Souza, B., Albert, I., Müller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. Association of the *APC* gene product with β-catenin. Science (Washington DC), *262:* 1731–1734, 1993.
28. Su, L-K., Vogelstein, B., and Kinzler, K. W. Association of the APC tumor suppressor protein with catenins. Science (Washington DC), *262:* 1734–1737, 1993.
29. Birchmeier, W., Weidner, K. M., Hülsken, J., and Behrens, J. Molecular mechanisms leading to cell junctions (cadherin) deficiency in invasive carcinomas. Semin. Cancer Biol., *4:* 231–239, 1993.

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