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Mechanisms of Radiation-Induced Neoplastic Transformation of Human Bronchial Epithelial Cells

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Carcinogenesis is a multistage process with sequences of genetic events that govern the phenotypic expression of a series of transformation steps that lead to the development of metastatic cancer. To better understand the mechanisms involved in human bronchial carcinogenesis induced by α particles from radon, we have developed a model of neoplastic transformation based on human papillomavirus-immortalized human bronchial epithelial (BEP2D) cells. Cells exposed to α particles become tumorigenic after progressing through a series of sequential stages including altered growth pattern, resistance to serum-induced terminal differentiation, agar-positive growth, tumorigenicity, and metastasis, with each step representing a necessary yet insufficient step toward the later, more malignant phase. Cell fusion studies indicated that the radiation-induced tumorigenic phenotype in BEP2D cells can be completely suppressed by fusion with nontumorigenic BEP2D cells. Several cellular differentiation and growth regulation genes such as *DCC* (deleted in colorectal cancer), *CDKN1A* (also known as $p21^{C1P1}$) and the gene that encodes DNA-PK were frequently found to be modulated in tumorigenic BEP2D cells and may be related to the process of carcinogenesis.

INTRODUCTION

Although radiation is a well-established human carcinogen and induces cancers in tissues of diverse histological origin, the mechanism(s) of radiation carcinogenesis is largely unknown. The carcinogenic risk for human epithelial cells after exposure to high-LET radiation has been estimated to be in the range of 10^{-12} per cell per gray based on breast cancer incidence among Japanese A-bomb survivors (*1*), an incidence that is too low to be reproduced in any laboratory setting. To better understand the genotypic and phenotypic changes associated with radon-induced lung cancer in humans, it would be ideal to use human bronchial cells to assess the various stages of transformation that lead to malignancies. However, no primary human cell model is currently available for this area of study because such cells have proven to be refractory to malignant transformation *in vitro* (*2*). One of the main reasons for this very low frequency (10^{-6} to 10^{-9}) is that human cells rarely undergo spontaneous immortalization (*3, 4*), which is an essential step for *in vitro* neoplastic transformation (*5, 6*). We have previously shown that human papillomavirus-immortalized human bronchial epithelial (BEP2D) cells can be malignantly transformed by a single 30-cGy dose of high-LET α particles at a frequency estimated to be $\sim 4 \times 10^{-7}$ after successive cultivation for 3 to 4 months postirradiation (*7, 8*). The immortalization step therefore increases the transformation of primary human epithelial cells by a million-fold. Although the incidence is still a factor of 10^3–10^4 lower than those obtained using established rodent cell models such as C3H 10T½ and NIH 3T3 cells, the use of immortalized human epithelial cells nevertheless makes it technically feasible to investigate the later stage of the process of neoplastic transformation.

A HUMAN BRONCHIAL CELL MODEL

BEP2D cells were initiated by lipofectin transfection of cloned full-length HPV-18 into normal bronchial epithelial cells obtained as an outgrowth of a bronchial explant (*9*). These cells are near diploid and are relatively stable genotypically over many passages (*7, 10*). Furthermore, they are anchorage-dependent and nontumorigenic in nude mice (*1, 7, 8*). The observation that BEP2D cells are nontumorigenic even in late passages suggests that abnormal TP53 and RB functions are not sufficient criteria for development of tumors and that additional genetic changes are needed. Transformed BEP2D cells progress through sequential stages including altered growth kinetics and anchorage-independent growth before becoming tumorigenic and producing progressively growing subcutaneous tumors upon inoculation into athymic nude mice. A few of these tumors also form metastatic colonies in lung and liver (*11*). It should be pointed out that, while the majority of agar-positive BEP2D clones were nontumorigenic, they all demonstrated the propensity to resist serum-induced terminal differentiation. In contrast, the majority of tumorigenic cell lines were agar-positive, suggesting that anchorage-independent growth is often a necessary but insufficient criterion for tumorigenicity. Cytogenetic and restriction fragment length polymorphism analysis indicated that many of the radiation-induced transformants had lost various chromosomal fragments including regions containing putative tumor sup-

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TABLE 1
Changes Associated with Radiation-Induced Tumorigenicity in BEP2D Cells

No mutation in any of the *RAS* oncogenes (7)
Absence of microsatellite mutator phenotype (*15*)
Loss of heterozygosity in polymorphic markers on chromosomes 8 and 14 (*10*)
Loss of chromosome Y (*10*)
Overexpression of cyclin D1 (*8*)
Expression of mutant TP53 with mutations in the *TP53* gene (exons 5, 6 and 8) only in secondary and metastatic tumors (*11*)
Telomerase activity was highest among the metastatic tumors (*16*)
Contain phosphorylated RB proteins (*8*)
Down-regulation of the cyclin-dependent kinase inhibitor CDKN1A

pressor genes such as those located on chromosomes 8 and 14 (*10*). It is clear from these studies that BEP2D cells exposed to α-particle radiation from radon undergo gradual, sequential changes of altered growth and anchorage-independent growth before becoming tumorigenic in nude mice. In addition, each preceding stage represents a necessary yet insufficient step toward the later, more malignant phase (*8, 12*).

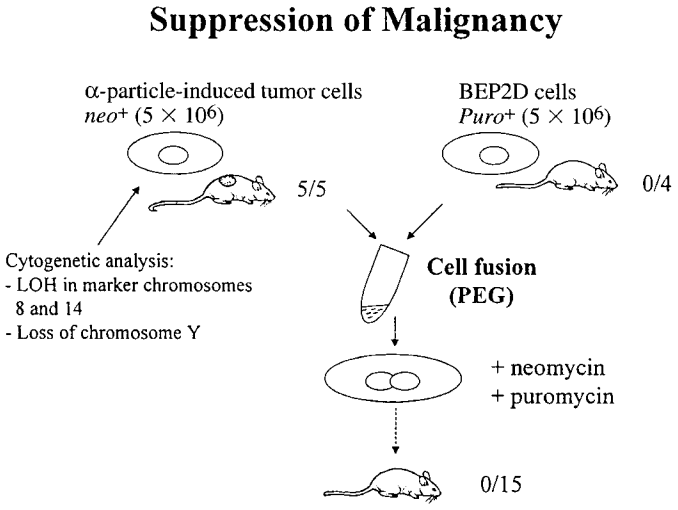

FIG. 1. Cell fusion approach for examination of the role of suppressor functions among tumorigenic BEP2D cells induced by α particles. The number of animals bearing tumors/the total number of animals injected for each group is shown.

TUMORIGENICITY IS A RECESSIVE PHENOTYPE

Table 1 lists all the currently known cytogenetic and molecular alterations associated with the tumorigenic phenotype in BEP2D cells induced by high-LET α particles. It is likely that multiple pathways are involved in the process of neoplastic transformation. The fact that high-LET radiation induces predominately multilocus deletions (*13, 14*) provides a mechanism for the potential loss of suppressor functions. To determine whether tumorigenicity of BEP2D cells behaves as a dominant or recessive trait, we carried out cell fusion studies as shown in Fig. 1. Tumorigenic BEP2D cells induced by a single 60-cGy dose of α particles were fused with control cells using polyethylene glycol. Briefly, 5×10^6 tumorigenic BEP2D cells infected with a pRC/CMV expression vector containing a *neo* gene were fused with an equal number of control BEP2D cells containing a p-Babe plasmid which is resistant to puromycin using 1 ml of prewarmed 50% PEG 1500. The PEG was added dropwise over 1 min to initiate the cell fusion process and was followed by the addition of 20 ml of culture medium over 6 min with constant agitation. The resulting fusion cells were then selected by incubation in medium containing both G418 and puromycin over a 12-day period. The fusion cells were expanded in culture and reinoculated into nude mice for expression of the tumorigenic phenotype. Controls involving the fusion of tumor–tumor and control–control cells were conducted similarly. Results of these fusion experiments demonstrated that the radiation-induced tumorigenic phenotype in BEP2D cells could be completely suppressed by fusion with nontumorigenic BEP2D cells. Furthermore, concurrent fusion of tumor cells with tumor cells resulted in tumorigenic hybrids, whereas fusion of wild-type BEP2D cells resulted in nontumorigenic hybrid clones. These data indicate that nontumorigenic BEP2D cells complement the loss of putative suppressor elements among tumorigenic cells and suggest loss of suppressor gene(s) as a likely mechanism of radiation carcinogenesis.

SEARCHING FOR TRANSFORMING GENES

The differential expression of known genes between tumorigenic BEP2D cells induced by α particles and control BEP2D cells was compared using a cDNA expression array (Clontech, Palo Alto, CA) as shown in Fig. 2. Briefly, total RNA from both tumorigenic and control BEP2D cells was isolated using Trizol Reagent (Gibco, Grand Island, NY). Then the isolate was treated with DNase I to remove any contaminating DNA from the RNA preparation as described (*1, 8*). PolyA$^+$ RNA was then isolated, and ^{32}P-labeled complex cDNA probes were generated by reverse transcription in the presence of ^{32}P-labeled dATP. The cDNA probes were then hybridized to the nylon membranes and the hybridization patterns were analyzed by autoradiography. The intensity of the signal, which corresponded to the expression levels of the individual genes, was quantified using phosphorimaging analysis. Overall, mRNA was prepared and analyzed at least three times from each tumor and control cell line examined.

As shown in Fig. 2, the Clontech cDNA expression array contains six quadrants which covers different categories of genes, including oncogenes, tumor suppressor genes, intracellular signal transduction modulators, DNA synthesis and repair genes, transcription factors, and receptor and growth factor genes. Each cDNA of the 588 preset genes was spotted in duplicate on the membranes. The hybridization signals appearing at the bottom of the array were various housekeeping genes intended to be used as positive controls. A series of 10 genes were identified to be differentially expressed between the two cell lines examined including the genes which code for the M-phase inducer pro-

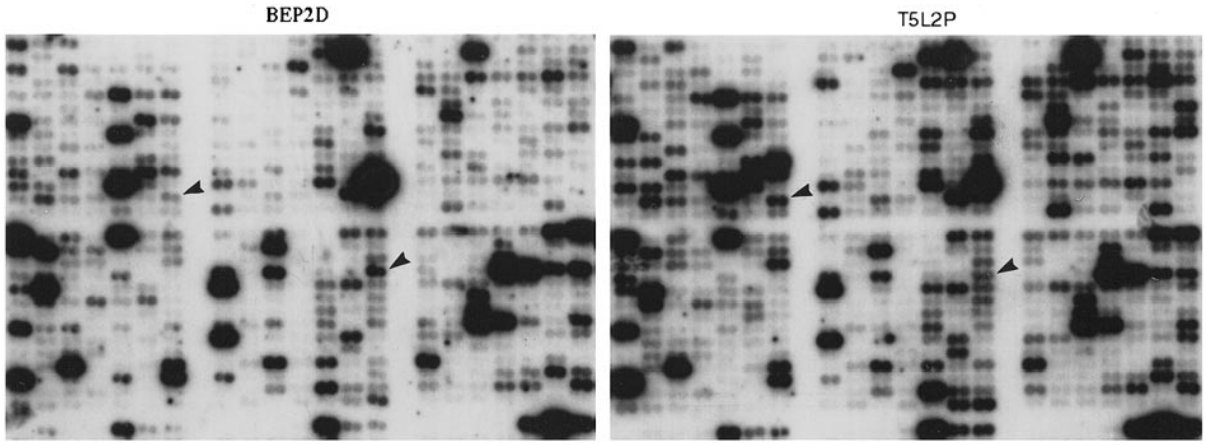

FIG. 2. Differential gene expression in a representative tumorigenic BEP2D cell induced by a single 60-cGy dose of α particles (T5L2P, right panel) relative to control BEP2D cells (left panel). The labeled cDNAs were hybridized to the array overnight at 65°C. The image was captured on X-ray film by exposure for 2 days at −80°C. Arrows indicate the location of the cDNA spots on the membrane for (1) CDC5B and (2) HSP27.

tein, CDC25B (number 1, arrow in upper left quadrant) and heat-shock protein 27 (number 2, arrow in upper middle quadrant, Fig. 2).

Since the incidence of false positives among cDNA expression analyses can be as high as 15–20%, it is essential to double-check the message level using Northern blotting. Figure 3 shows the expression level of *CDKN1A* (also known as $p21^{CIP1}$) among control and tumorigenic BEP2D cells induced by a single 60-cGy dose of α particles. In control (lane 1) and irradiated BEP2D cells 1 week postirradiation (lane 2), the expression level of *CDKN1A* was about twofold higher than that in the irradiated population prior to their injection into nude mice (lane 3). Among tumorigenic BEP2D cells, the average expression level of *CDKN1A* was about fourfold lower than that of the control BEP2D cells (lanes 4 through 8). It should be noted that equivalent quantities of mRNA based on the expression level of control β-actin were loaded into each lane. Table 2 lists the differentially expressed genes and their relative expression levels pooled from 5–8 tumorigenic BEP2D cell lines induced by α particles. Expression levels of the individual genes were compared relative to the level of the β-actin gene from the same membrane. Among the 10 genes initially identified as differentially expressed, *MYC* and fibronectin could not be confirmed when subsequently analyzed by Northern blotting. Several cell growth and differentiation genes were found to be down-regulated in the tumorigenic BEP2D cells relative to their corresponding controls. These included the *DCC* tumor suppressor gene, the DNA-dependent protein kinase gene (*PRKDC*), the differentiation marker cytokeratin CK14, and the cell cycle modulator *CDKN1A* gene.

CDKN1A is a cyclin-dependent kinase inhibitor that can effectively inhibit CDK2, CDK4 and CDK6 and is capable of inducing cell cycle arrest in G_1 phase when it is overexpressed. Although CDKN1A is induced by TP53 in response to radiation, CDKN1A can be expressed at high levels in TP53-negative cells, indicating the existence of TP53-independent mechanisms to regulate CDKN1A expression (*17*). There is evidence that CDKN1A may be involved in the G_2-phase checkpoint regulation in addition to exerting negative control of the G_1/S-phase transition (*18*). The down-regulation of CDKN1A has been shown to increase CDC2 and CDK2 activity and promote cell cycle progression and cell proliferation (*19*). Reduced levels of CDKN1A protein have been reported in many human can-

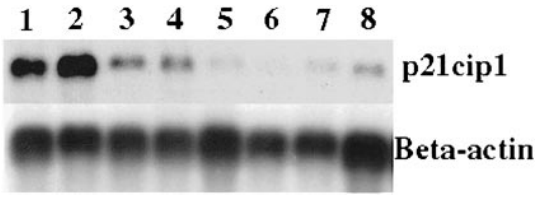

FIG. 3. Northern blot analysis showing the relative levels of the mRNA of CDKN1A (p21cip1) in control BEP2D cells (lane 1), irradiated BEP2D cells 1 week postirradiation (lane 2), irradiated cells just before inoculation into nude mice (lane 3), and five representative tumor cell lines (lanes 4 through 8). The RNA blot was hybridized to ^{32}P-labeled human CDKN1A probe. The relative abundance of RNA per lane was judged to be similar based on the β-actin level.

TABLE 2
List of Differentially Expressed Genes between Tumorigenic and Control BEP2D Cells Based on mRNA Values

Gene	Expression level
DCC tumor suppressor	↓ 2.1 ± 0.2
DNA-dependent protein kinase	↓ 2.2 ± 0.5
Heat-shock protein 27	↓ 4.9 ± 2.3
Cytokeratin 14	↓ 5.0 ± 0.8
Alpha-catenin	↓ 2.2 ± 0.5
CDKN1A	↓ 4.4 ± 2.1
Glutathione-*S*-transferase	↑ 2.1 ± 0.4
CDC25B (M-phase inducer)	↑ 3.3 ± 1.2
MYC	No change
Fibronectin	No change

cers and may contribute to malignant progression (*20*). On the other hand, overexpression of CDKN1A has been shown to inhibit the growth of several tumor cell lines (*21*). In the present study, the level of expression of *CDKN1A* mRNA was found to be down-regulated twofold in radiation-treated late-passage cells and fourfold in tumorigenic cells even though TP53 was already inactivated by the presence of E6 oncoprotein in BEP2D cells. These data suggest the presence of a TP53-independent regulatory pathway of CDKN1A expression that is likely to play an important role in malignant progression induced by radiation.

DCC (deleted in colorectal cancer) is a candidate tumor suppressor gene that is postulated to function as a transmembrane receptor that belongs to the immunoglobulin superfamily (*22*). Frequent allelic deletions and loss of its expression and somatic mutations have been seen not only in colorectal cancer but also in a number of other cancers as well (*23*). Inactivation of the *DCC* gene may enhance the ability of tumor cells to invade and metastasize (*24*). Some of the strongest evidence supporting a role for *DCC* as a tumor suppressor gene comes from studies showing a suppression of tumorigenicity in nude mice by expression of full-length, but not truncated, *DCC* in nitrosomethylurea (NMU)-transformed keratinocytes lacking *DCC* expression (*25*). When *DCC* cDNA is transfected into tumor cells, apoptosis and G_2/M-phase cell cycle arrest are induced by caspase-3 activation and inhibition of CDK1, suggesting a possible mechanism by which *DCC* suppresses tumorigenesis (*26*).

The CDC25 family of mammalian phosphatases includes three homologues, CDC25A, CDC25B and CDC25C, which can activate cyclin-dependent kinases by removing inhibitory phosphates from tyrosine and threonine residues. The CDC25 functions at the G_1/S-phase and G_2/M-phase borders (*27*). The overexpression of CDC25B was observed in many breast, head and lung cancers (*28*). The *CDC25B* cooperates with *HRAS* or loss of RB1 in oncogenic transformation, and high expression of *CDC25B* in a portion of primary breast cancers correlates with less favorable prognosis and poor survival (*29*). Reduced *CDC25B* mRNA level and deregulated protein expression by antisense oligonucleotides result in S-phase delay and antiproliferative effects in synchronized HeLa cells (*30*). This evidence indicates that *CDC25B* possesses oncogenic properties and is consistent with our observation that *CDC25B* was up-regulated threefold in tumor cells, suggesting that an overexpression of *CDC25B* may play an important role in radiation carcinogenesis.

Identification of the stepwise molecular alterations in carcinogenesis is one of the major goals in cancer research. The cDNA array provides a useful method to screen differentially expressed genes between control and tumorigenic BEP2D cells. Although our present study identified a number of differentially expressed genes in tumorigenic and control BEP2D cells, the nature of the interaction of these genes in modulating radiation-induced tumorigenesis is not yet clear. It is likely that a concerted effort by many genes rather than loss of a single suppressor function is critical for the tumorigenic phenotype. For example, a reduced expression level of *PRKDC* may contribute to genetic instability of transformed BEP2D cells (*31*). Subsequent down-regulation of the E-cadherin-catenin complex may enhance invasiveness of transformed cells (*32*). Together with the cytokeratin 14 gene, whose expression is down-regulated by fivefold in tumorigenic cells, these altered gene expressions may serve as a useful biomarker for the transformed phenotype of BEP2D cells. Understanding the genes and their products that regulate tumor progression and invasiveness are of critical importance not only for a better understanding of the mechanism of radiation carcinogenesis but also as an aid in the eventual design of novel prognostic and therapeutic approaches.

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REFERENCES

1. T. K. Hei, C. Q. Piao, T. Sutter, J. C. Willey and K. Suzuki, Cellular and molecular alterations in human epithelial cells transformed by high LET radiation. *Adv. Space Res.* **18**, 137–148 (1996).
2. J. S. Rhim and A. Dritschilo, Neoplastic transformation in human cell system—an overview. In *Neoplastic Transformation in Human Cell Culture: Mechanisms of Carcinogenesis* (J. S. Rhim and A. Dritschilo, Eds.), pp. xi–xxxi. Humana Press, Totowa, NJ, 1991.
3. J. C. Barrett and P. O. Tso, Relationship between somatic mutation and neoplastic transformation. *Proc. Natl. Acad. Sci. USA* **75**, 3297–3301 (1978).
4. J. W. Shay, B. A. Van der Haegen, Y. Ying and W. E. Wright, The frequency of immortalization of human fibroblasts and mammary epithelial cells transfected with SV40 large T antigen. *Exp. Cell Res.* **209**, 45–52 (1993).
5. M. Namba, K. Mihara and K. Fushimi, Immortalization of human cells and its mechanisms. *Crit. Rev. Oncogen.* **7**, 19–31 (1996).
6. T. M. Bryan and R. R. Reddel, SV-40 induced immortalization of human cells. *Crit. Rev. Oncogen.* **5**, 331–357 (1994).
7. T. K. Hei, C. Q. Piao, J. C. Willey, S. Thomas and E. J. Hall, Malignant transformation of human bronchial epithelial cells by radon-simulated alpha particles. *Carcinogenesis* **15**, 431–437 (1994).
8. T. K. Hei, C. Q. Piao, E. Han, T. Sutter and J. C. Willey, Radon induced neoplastic transformation of human bronchial epithelial cells. *Radiat. Oncol. Invest.* **3**, 398–403 (1996).
9. J. C. Willey, A. Broussoud, A. Sleemi, W. P. Bennett, P. Cerutti and C. C. Curtis, Immortalization of human bronchial epithelial cells by papillomaviruses 16 or 18. *Cancer Res.* **51**, 5370–5377 (1991).
10. D. Weaver, T. K. Hei, B. Hukka, J. A. McRaven and J. C. Willey, Cytogenetic and molecular analysis of tumorigenic human bronchial epithelial cells induced by alpha particles. *Carcinogenesis* **18**, 1251–1257 (1997).
11. C. Q. Piao, J. C. Willey and T. K. Hei, Alterations of p53 in tumorigenic human bronchial epithelial cells correlate with metastatic potential. *Carcinogenesis* **20**, 1529–1533 (1999).
12. T. K. Hei, C. Q. Piao, J. C. Willey and E. J. Hall, Genomic instability and tumorigenic induction in immortalized human bronchial epithelial cells by heavy ions. *Adv. Space Res.* **22**, 1699–1707 (1999).

13. H. H. Evans, Cellular and molecular effects of radon and other alpha particles emitters. *Adv. Mutagen. Res.* **3**, 29–57 (1991).

14. L. X. Zhu, C. A. Waldren, D. Vannais and T. K. Hei, Cellular and molecular analysis of mutagenesis induced by charged particles of defined LET. *Radiat. Res.* **145**, 251–259 (1996).

15. D. B. Xu, H. Feng, T. K. Hei, C. Q. Piao and S. M. Kahn, Absence of the microsatellite mutator phenotype in human bronchial epithelial cells transformed by alpha particles. *Int. J. Oncol.* **10**, 921–925 (1997).

16. T. J. Pandita, E. J. Hall, T. K. Hei, M. A. Piatyzsek, W. E. Wright, C. Q. Piao, R. K. Pandita, J. C. Willey, C. R. Geard and J. W. Shay, Chromosome end to end associations and telomerase activity during cancer progression in human cells after treatment with alpha particles simulating radon progeny. *Oncogene* **13**, 1423–1430 (1996).

17. J. W. Harper, G. R. Ademi, N. Wei, K. Veyomarsi and S. J. Elledge, The p21 cdk-interacting protein cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. *Cell* **75**, 805–806 (1993).

18. V. Dulic, G. H. Stein, D. F. Far and S. I. Reed, Nuclear accumulation of p21cip1 at the onset of mitosis: A role at the G_2/M-phase transition. *Mol. Cell Biol.* **18**, 546–557 (1998).

19. D. L. Crow and C. F. Shuler, Increased cdc2 and cdk2 kinase activity by retinoid X receptor gamma-mediated transcriptional down-regulation of the cyclin-dependent kinase inhibitor p21Cip1/WAF1 correlates with terminal differentiation of squamous cell carcinoma lines. *Cell Growth Differ.* **9**, 619–627 (1998).

20. M. Pinyol, L. Hernandez, M. Cazorla, M. Balbin, P. Jares, P. L. Fernandez, E. Montserrat, A. Cardesa, C. Lopezotin and E. Campo, Deletions and loss of expression of p16INK4a and p21Waf1 genes are associated with aggressive variants of mantle cell lymphomas. *Blood* **89**, 272–280 (1997).

21. W. S. el-Deiry, T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein, WAF1, a potential mediator of p53 tumor suppressor. *Cell* **75**, 817–825 (1993).

22. E. R. Fearon and W. E. Pierceall, The deleted in colorectal cancer (DCC) gene: A candidate tumour suppressor gene encoding a cell surface protein with similarity to neural cell adhesion molecules. *Cancer Surv.* **24**, 3–17 (1995).

23. K. R. Cho and E. R. Fearon, DCC: linking tumor suppressor genes and altered cell surface interactions in cancer? *Curr. Opin. Genet. Dev.* **5**, 72–78 (1995).

24. K. Ookawa, M. Sakamota, S. Hirohashi, Y. Yoshida, T. Sugimura, M. Tetada and J. Yokota, Concordant p53 and DCC alterations and allelic losses on 13q and 14q associated with liver metastases of colorectal carcinoma. *Int. J. Cancer* **53**, 382–387 (1993).

25. A. J. Klingelhutz, L. Herick, K. R. Cho and J. K. McDougall, The *DCC* gene suppresses the malignant phenotype of transformed human epithelial cells. *Oncogene* **10**, 1581–1586 (1995).

26. Y. Q. Chen, J. T. Hsieh, F. Yao, B. L. Fang, R. Pong, S. C. Cipriano and F. Krepulat, Induction of apoptosis and G_2/M cell cycle arrest by DCC. *Oncogene* **18**, 2747–2754 (1999).

27. K. Galaktionov and D. Beach, Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: Evidence for multiple roles of mitotic cyclins. *Cell* **67**, 1181–1194 (1991).

28. W. Wu, Y. H. Fan, B. L. Kemp, G. Walsh and L. Mao, Overexpression of cdc25A and cdc25B is frequent in primary non-small cell lung cancer but is not associated with overexpression of c-myc. *Cancer Res.* **58**, 4082–4085 (1998).

29. K. Galaktionov, A. K. Lee, J. Eckstein, G. Draetta, J. Meckler, M. Loda and D. Beach, CDC25 phosphatases as potential human oncogenes. *Science* **269**, 1575–1577 (1995).

30. P. A. Garner-Hamrick and C. Fisher, Antisense phosphorothioate oligonucleotides specifically down-regulate cdc25B causing S-phase delay and persistent antiproliferative effects. *Int. J. Cancer* **76**, 720–728 (1998).

31. M. Lun, R. L. Wells, S. Lang, N. Chawapun and M. M. Elkind, the neoplastic transformation of SCID cells by radiation. *Radiat. Res.* **152**, 180–189 (1999).

32. C. Xiangming, S. Hokita, S. Natsugoe, G. Tanabe, M. Baba, S. Takao, K. Kuroshima and T. Aikou, Cooccurrence of reduced expression of alpha-catenin and overexpression of p53 is a predictor of lymph node metastasis in early gastric cancer. *Oncology* **57**, 131–137 (1999).