Inhibiting tumorigenic potential by restoration of p16 in nasopharyngeal carcinoma

GL Wang, KW Lo, KS Tsang, NYF Chung, YS Tsang, ST Cheung, JCK Lee and DP Huang

Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong

Summary The p16 gene, encodes a key checkpoint protein p16 in the cell cycle, has been reported inactivation in a wide variety of human cancers. We have previously demonstrated high frequency of p16 alterations in primary nasopharyngeal carcinoma (NPC), xenografts and cell lines. The finding implied that inactivation of the p16 gene may play an important role in the NPC development. To investigate the tumour suppressor function of p16 in NPC, we transfected p16-deficient NPC cell line, NPC/HK-1, with a wild-type p16 expression construct, and evaluated growth and tumorigenic properties of the clones stably expressing exogenous p16. Expression of the exogenous wild-type p16 significantly inhibited cell growth by more than 70% when compared to that of the parental and empty vector-transfected cells. This growth inhibition was attributable to a significant proportion of p16-expressing cells arrested at G1 phase in the cell cycle as revealed by flow cytometric analysis. By anchorage-independent colony forming assay, we found that the ability to form colonies in soft agar was highly reduced in cells expressing p16. NPC/HK1 cells expressing functional p16 also showed suppressed tumorigenicity in athymic nude mice. Taken together, our results provide strong evidence for a tumour suppressor role of p16 in NPC.

Keywords: nasopharyngeal carcinoma; p16; tumour suppressor; gene transfer

Like most solid tumours, the tumorigenesis of nasopharyngeal carcinoma (NPC) involves accumulation of multiple genetic alterations. Overexpression of several proto-oncogenes Bcl-2, c-Myc and Ras has been reported in this tumour (Lu et al, 1993; Porter et al, 1994). Our previous studies demonstrated that frequent allelic losses at chromosomes 3p, 9p, 11q and 14q were found in NPC(Huang, 1991, 1994; Hui, 1996; Cheng et al, 1997). Homozygous deletion in 9p21–22 has been detected both in NPC primary tumours and xenografts (Huang et al, 1994; Lo et al, 1995). Recent studies have localized three INK4 family genes, p16 (CNKN2A, MTS1, INK4A), p15 (CNKN2B, INK4B) and p19 (ARF) to this affected region (Kamb et al, 1994; Hannon and Beach, 1994; Nobori et al, Chan et al, 1995). Among these genes, high incidence of p16 gene alterations (77.1%), either due to homozygous deletion or aberrant methylation resulting in loss of p16 expression, has been observed in the primary tumours (Lo, 1995; Lo et al, 1996). It is likely that p16 is the primary target for inactivation in this region. Frequent absence of the p16 protein has also been reported (Gulley et al, 1998). Detection of high frequency of altered p16 gene in NPC stands it out as the most common genetic abnormality found in this cancer.

The progression of a normal cell from G1 into S phase in a cell cycle is regulated by the cyclin-dependent kinases (cdk) 4/6 and cyclin D1 complex through phosphorylation of the retinoblastoma protein (pRb) at the late G1 phase (Hinds et al, 1992; Kato et al, 1993). By competing with cyclin D1 for binding to cdk4/6, the p16 protein inhibits phosphorylation of pRb and arrests cells at the G1 phase. Inactivation of p16 may lead to persistent pRb phosphorylation and, therefore, resulting uncontrolled cell proliferation.

The current study aimed to investigate the tumour suppressor role of p16 in NPC. We introduced a wild-type p16 cDNA expression construct into a p16-deficient NPC cell line NPC/HK-1 and examined for growth and tumorigenic parameters of the resultant transfected cells. Our data demonstrated that restoration of p16 expression in NPC/HK1 cells suppressed growth by arresting cells at G1 phase and inhibited tumorigenicity in athymic nude mice.

**MATERIALS AND METHODS**

**Cell line and transfection**

The human cell line NPC/HK-1 was derived from a well-differentiated NPC tumour (Huang et al, 1980). No p16 protein is found in this cell line as one allele of the p16 gene is deleted and the other shows mutation at the splice site of exon 2 (Lo et al, 1995). NPC/HK-1 also expresses a mutant p53 (Spruck et al, 1992) and a functional pRb. HeLa cells with intact p16 was used as positive control. Cells were grown in RPMI-1640 medium (Sigma Chemical Company, St Louis, MO, USA) supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY, USA).

The pCMV-p16 plasmid contains a full-length wild-type p16 gene inserted downstream of a CMV promoter and a neomycin resistance selectable marker (Liggett et al, 1996; a gift from Professor David Sidransky, Johns Hopkins University, USA). The pCMV empty vector and the pCMV-p16 construct were transfected into NPC/HK-1 cells and HeLa cells separately using Lipofectamine reagent (Gibco-BRL, Grand Island, NY, USA) according to manufacturer’s protocol. Transfected cells were selected in 300 µg ml⁻¹ G418 (Gibco-BRL, Grand Island, NY, USA) for 4–5 weeks. Individual G418-resistant colonies were then picked using the trypsin-soaked filter paper discs method and expanded for further analyses.
Polymerase chain reaction
In order to detect the presence of pCMV-p16 in the transfected cells, the primers S9 and S13 flanking exon 1 to 3 of the p16 gene were used to amplify the exogenous p16 cDNA sequence as described (Lo et al, 1996).

Northern blot analysis
Total RNA was isolated from cells using TRizol reagent (Gibco-BRL, Grand Island, NY, USA) according to manufacturer’s instruction. Twenty micrograms of total RNA was subjected to electrophoresis in a 1.5% formaldehyde-containing denaturing agarose gel followed by capillary transfer onto the Hybond-N membrane (Amersham, Little Chalfont, UK). A full-length p16 cDNA probe was labelled with α-32P[dCTP] using the Rediprime DNA labelling system (Amersham, Little Chalfont, UK). The blot was hybridized with the p16 probe using Rapid Hyb buffer (Amersham, Little Chalfont, UK). After stringency washing, the blot was exposed to Kodak X-OMAT K film (Kodak, Rochester, NY, USA). To normalize the levels of transcripts, the blot was stripped and rehybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe.

Western blot analysis
Protein extracts were prepared according to Pagano et al (1993). Fifty micrograms of protein were separated on a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electro-transferred onto the ECL-NC membrane (Amersham, Little Chalfont, UK). The blots were blocked with phosphate-buffered saline (PBS) containing 5% non-fat milk and 0.1% Tween-20 for 30 min at room temperature and treated with rabbit anti-human p16 polyclonal antibody (1:1500; PharMingen, San Diego, CA, USA) for 2 h at room temperature. After PBS wash, the blots were incubated with a peroxidase-conjugated anti-rabbit secondary antibody (1:2000; Dako, Kyoto, Japan) for 1 h. Antigen-antibody complexes were detected by the chemiluminescence reagent (Amersham, Little Chalfont, UK). The expression of Rb and cyclin D1 were detected by human monoclonal antibodies of Rb (G3-245, PharMingen, San Diego, CA, USA) and cyclin D1 (G124-326, PharMingen, San Diego, CA, USA) respectively. To ensure equal loading of protein extracts, a parallel gel was stained with Coomassie brilliant blue G250 (Sigma Chemical Company, St Louis, MO, USA).

Cell growth and cell cycle analyses
Growth curves were constructed by cell counting over a set period of cultivation. Cells were seeded onto a 24-well plate at 2 x 10^4 cells per well and grew in medium. A total of 300 µg ml⁻¹ G418 was added to the medium for the transfected cells. Culture medium was changed every 2 days and the number of cells was counted consecutively for 7 days. Each experiment was done in triplicate.

For flow cytometry analysis, cells (~5 x 10⁶) were harvested by trypsinization, washed with Hank’s balanced salt solution (HBSS, Sigma Chemical Company, St Louis, MO, USA), fixed in 70% ethanol at 4°C for 2 h, then allowed to pass through 40 µm cell strainer (Falcon, Becton Dickinson, USA). After washing with HBSS and adjusting the cell number to 1 x 10⁶ ml⁻¹ in HBSS supplying with propidium iodide (50 µg ml⁻¹), the cellular DNA content was assessed by the System II software in a Coulter EPICS XL MCL flow cytometer (Coulter Corporation, Miami, FL, USA) and the cell cycle distribution data were analysed by the Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

Anchorage-independent growth assay
Five hundred exponentially growing cells were suspended in 2 ml medium containing 0.35% low-melting temperature (LMT) agarose gel (FMC, Rockland, ME, USA) and overlaid onto a layer of 2 ml 0.6% LMT agarose gel/RPMI-1640 of each well of a 6-well plate. For the p16-transfected cells, the agarose gel/medium containing 300 µg ml⁻¹ G418 was used. After 3 weeks, colonies composed of at least 16 cells were counted under an inverted microscope.

Tumorigenicity assay
Balb/c nude mice of 7- to 8-week-old were used in the tumorigenicity assay. Cells (3 x 10⁶) suspended in 0.5 ml RPMI-1640 medium were injected subcutaneously into the back of each nude mouse. Tumour growth was monitored by measuring the size of tumours using a caliper.

RESULTS
Expression of exogenous wild-type p16
We transfected a full-length wild-type p16 expression cassette into p16-null NPC/HK-1 cells and isolated colonies that were resistant to G418. Polymerase chain reaction (PCR) analysis demonstrated that six clones (N21, N24, N50, N52, N55, N104) contained the p16 cDNA fragment, which was absent from parental cells and cells transfected with empty vector (Figure 1A). Northern blot analysis revealed the presence of a hybridizing band in these six p16-transfected clones, whereas parental cells showed no hybridization signal to the p16 probe (Figure 1B). The size of hybridizing band of the exogenous p16 transcript was greater than that of the endogenous p16 transcript, as revealed in HeLa and HeLa p16-transfected cells. In addition to the band corresponded to the endogenous p16 transcript, the larger hybridizing band of the exogenous p16 transcript showed the same size as those bands seen in p16-transfected NPC/HK-1 cells. After normalization with the GAPDH signals, we found the expression levels of exogenous p16 transcripts varied among the six p16-transfected NPC/HK-1 clones. Clone N21 expressed the highest level of p16 transcripts. Western blot analysis was then performed to assess the expression of p16 protein. Using a p16-specific antibody, we detected a band of 16 kDa, characteristics of human p16, in protein extracts of the six clones (Figure 1C). In contrast, parental and empty vector-transfected cells showed no expression of the pRb and cyclin D1 proteins. Using a p16-specific antibody, we detected a band of 16 kDa, characteristics of human p16, in protein extracts of the six clones (Figure 1C). In contrast, parental and empty vector-transfected cells showed no expression of the pRb and cyclin D1 proteins. Additionally, we detected a band of 16 kDa, characteristics of human p16, in protein extracts of the six clones (Figure 1C). In contrast, parental and empty vector-transfected cells showed no expression of the pRb and cyclin D1 proteins.
Effect of p16 on cell growth

To evaluate the effects of wild-type p16 expression on growth, we examined the growth rates of the p16-transfected clone N21, parental NPC/HK-1 cells and the empty vector-transfected cells by cell proliferation assay. There was no significant difference in the growth rate between NPC/HK-1 parental and empty vector-transfected cells (Figure 2). However, the proliferate ability of clone N21 was reduced and these cells grew at a slower rate. On day 7, growth of the p16-transfected clone N21 was inhibited by 70% relative to the empty vector-transfected cells.

Flow cytometric analysis was used to assess the effect of p16 on the cell cycle of the transfected cells (Figure 3). No significant difference in cell cycle distributions were detected in parental and empty vector-transfected cells. In contrast, the proportion of cells in the S phase were reduced by more than four times in clone N21 (5.8%) when compared with those in the vector-transfected (25.2%) and parental cells (30.5%). A corresponding increase in cell numbers in G1 phase by 22% was also observed in N21 cells (88.8%) comparing with those in the controls (66.3% and 66.4%). Our results indicate that restoration of functional p16 in NPC cells suppressed cell growth by arresting cells in the G1 phase.

Anchorage-independent growth and tumorigenicity assays

We analysed the anchorage-independent growth transforming potential of transfected cells by their ability to form colonies in soft agar. The number and size of the colonies were comparable in parental and empty vector-transfected cells. However, p16-transfected clone N21 had reduced ability to grow in soft agar and the colonies formed were much smaller (16–25 cells per colony) than the colonies (> 200 cells per colony) formed by control cells. The colony forming efficiency of the p16-transfected cells were about tenfold lower when compared to those of the parental and vector-transfected cells. These data indicate that replacement of the p16 gene reduced transforming potential in NPC cells.

Table 1: Anchorage-independent growth in vitro and tumorigenicity in athymic nude mice by functional restoration of p16 expression in NPC/HK-1 cells

| Cell type                  | No. of colony | Nude mice | Tumour volume (mm³)a |
|----------------------------|---------------|-----------|----------------------|
| Parental                  | 34            | A         | 530                  |
| Empty vector-transfected  | 32            | A         | 800                  |
| p16-transfected           | 3             | A         | 0b                   |
|                            |               | B         | 1200                 |

At 1 month post-inoculation. *Tumour was examined over a 10-month interval.

Figure 2: Growth curves of NPC/HK-1 cells. The mean of cell counts from three independent experiments were plotted. Cell growth of p16-expressing N21 cells was reduced by 70% as compared to those of parental and empty vector-transfected cells.

Table 1

| Cell type                  | No. of colony | Nude mice | Tumour volume (mm³)a |
|----------------------------|---------------|-----------|----------------------|
| Parental                  | 34            | A         | 530                  |
| Empty vector-transfected  | 32            | A         | 800                  |
| p16-transfected           | 3             | A         | 0b                   |

*(Tumour size was measured 1 month post-inoculation. *Tumour was examined over a 10-month interval.)*

Figure 1: Expression of exogenous p16 in p16-null NPC/HK-1 cells. (A) Demonstration of the presence of p16 cDNA fragment in transfected cells by PCR. The expected size of the PCR product was 485 bp. (B) Northern blot analysis of the p16 expression. Exogenous p16 transcripts were detected in cells transfected with p16. (C) Western blot analysis of p16 protein expression. Clones that expressed exogenous p16 transcripts also expressed the p16 protein.
The p16-transfected cells as well as the parental and empty vector-transfected cells were injected into nude mice to test for their tumorigenicity. About 10 days post-inoculation, sizable tumours were observed in all nude mice injected with parental or vector-transfected cells. Table 1 depicts the growth of tumours in nude mice, 1 month after inoculation. No tumour was observed in the nude mice injected with the p16-expressing N21 cells over a period of 10 months. Our results demonstrate that re-expression of wild-type p16 in NPC cells suppressed tumorigenicity of the malignant carcinoma cells.

DISCUSSION

It has been recognized that nasopharyngeal carcinoma, like other solid tumours, develops and progresses as a consequence of multiple genetic alterations (reviewed in Lo, 1997). Among these genetic changes, p16 inactivation stands out in its high frequency of occurrence in this cancer. As a critical tumour suppressor, p16 acts as an inhibitor of cdk4/cdk6 and can block the cyclin D1-dependent phosphorylation of the Rb protein. Loss of p16 protein coupled with phosphorylation of the Rb protein releases the E2F transcription factors. This signals a cell to enter into the S phase, and initiates uncontrolled cell proliferation. In this study, we have demonstrated that restoration of the wild-type p16 activity in the p16-null NPC cells caused marked growth suppression and loss of tumorigenic potential. Our results indicate that loss of the p16 gene function is an important molecular event in the tumorigenesis of NPC.

The well-characterized NPC cell line NPC/HK-1, which expresses functional Rb protein and contains no wild-type p16 gene, was selected for the transfection study with pCMV-p16 plasmid. To investigate the tumour suppressive activity of p16 in the NPC cells, the stable transfected cells consistently expressing exogenous wild-type p16 protein were isolated. Although many groups have reported that exogeneous p16 protein has morphological effect on several types of cancer cells (Shapiro et al, 1995; Castellano et al, 1997), we did not observe obvious morphological changes in the exogeneous p16-expressing NPC cells. It is likely that the expression of the exogenous p16 did not alter the differentiation in these cells. The highest level of p16 expression among these clones was found in clone N21. Study of clone N21 showed that replacement of the wild-type p16 gene in NPC/HK-1 resulted in marked growth suppression. We have also performed apoptosis analysis and found that the number of apoptotic cells did not increase after restoration of the wild-type p16 in the NPC cells (data not shown). The result indicated that reduction of cell growth was not due to apoptosis induced by p16. Flow cytometry analysis of the cell cycle in N21 showed that the expression of the exogeneous p16 inhibited growth by inducing a G1 arrest of the cell cycle in these NPC cells. Our data clearly demonstrated the growth-inhibitory role of the p16 gene in NPC. In addition, the exogeneous p16 expression in the p16-null NPC cells also inhibited the tumorigenic potential. By anchorage-independent colony forming assay, we found that the ability to form colonies in soft agar was highly reduced in the cells that expressed exogenous p16. In vivo tumorigenic study further demonstrated that these p16-expressing NPC cells failed to grow tumours in nude mice when compared with the control cells, for a period as long as 10 months follow-up.

Restoration of the p16 gene into other cancer cell types has been performed previously and demonstrated similar effects on the cancer cells. The effect of replacement of the p16 gene has been shown to be dependent on the recipient cells. Introduction of the p16 into glioma cells (Fueyo et al, 1996) and leukaemic cells (Quesnel et al, 1996) have been shown to lead to cell growth inhibition by inducing G1 phase accumulation in the cell cycle and reduction in anchorage-independent growth ability. In melanoma cells, only cell growth inhibition and morphologic changes induction had been reported (Castellano et al, 1997). The effect of inhibiting tumorigenicity in athymic mice was also observed in the p16-restored colon carcinoma cells (Spillare et al, 1996) and non-small cell lung carcinoma cells (Jin et al, 1995).

Our findings have, for the first time, directly proved the critical suppressor role of the p16 gene in the progression of NPC and suggested its potential adequacy in gene replacement therapy. As the molecular basis of NPC tumorigenesis is still not clear and only a few target genes identified for the development of gene therapy, strategies based on p16 replacement may be one of the
effective molecular approaches in the treatment of NPC. It has been reported that the reintroduction of the wild-type p53 protein in some NPC cell lines with p53 mutations showed cytotoxic effect on the tumour cells. The high level expression of exogenous p53 protein has led to tumour cell death, mediated through apoptotic pathways in NPC cells (Li et al., 1997). The true usefulness of this gene therapy strategy remains to be tested as majority of NPC primary tumours are found to contain the wild-type p53 gene. By mono-chromosome transfer, Cheng et al. (1998) has demonstrated that chromosome 3p21.3, frequently deleted in this cancer, processes tumour suppressor function in NPC cells. The target tumour suppressor gene(s) in this region has, however, not yet been identified. Our present findings, both in vitro and in vivo, demonstrated a significant anti-tumour effect of the p16 replacement against NPC. Some reports indicated that restoration of p16 correlated with increased radiosensitivity (Miyakoshi et al., 1997). The potential of adenovirus-mediated p16 gene transfer to NPC cells would be a logical step for the planning of our next investigation. Since the high frequency of p16 gene inactivation in NPC, the therapy strategies based on p16 replacement may be one of the most effective molecular approaches in the treatment of NPC.

ACKNOWLEDGEMENTS

We thank Professor D. Sidransky of The Johns Hopkins University, USA for providing the pCMV-p16 plasmid and Mr Jesse C S Pang for suggestions and advising the edition of this manuscript. The project was funded partly by the Research Grant Council of Hong Kong (CUHK 261/96M and CUHK 4284/98M).

REFERENCES

Castellano M, Gabrielli BG, Hussussian CJ, Dracopoli NC and Hayward NK (1997) Restoration of CDKN2A into melanoma cells induces morphologic changes and reduction in growth rate but not anchorage-independent growth reversal. J Invest Dermatol 109: 61–68

Chen FKM, Zhang J, Chen L, Shapiro DN and Winoto A (1995) Indentification of human/mouse p19, a novel cdk4/cdk6 inhibitor with homology to p16ink4. Mol Cell Biol 15: 2682–2688

Cheng RYS, Lo KW, Huang DP and Tsao SW (1997) Loss of heterozygosity on the long arm of chromosome 11 in nasopharyngeal carcinoma. Jpn J Cancer Res 88: 2682–2688

Cheng RYS, Lo KW, Huang DP and Tsao SW (1997) Loss of heterozygosity on the long arm of chromosome 11 in nasopharyngeal carcinoma. Int J Oncol 10: 1047–1050

Chen Y, Poulos NE, Lung ML, Hampton G, Ou B, Lerman MI and Stanbridge EJ (1998) Functional evidence for a nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. Proc Natl Acad Sci USA 95: 3042–3047

Fuego J, Gomez-Manzano C, Yung WKA, Clayton GL, Liu TJ, Bruner J, Levin VA and Kyritsis AP (1996) Adenovirus-mediated p16/CDKN2 gene transfer induces growth arrest and modifies the transformed phenotype of glioma cells. Oncogene 12: 103–110

Gilley ML, Nicholls JM, Schneider BG, Amin MB, Ro JY and Geradts J (1998) Nasopharyngeal carcinomas frequently lack the p16/MTS1 tumor suppressor protein but consistently express the retinoblastoma gene product. Am J Pathol 152: 865–869

Hannon GJ and Beach D (1999) p53[QD] is a potential effector of cell cycle arrest mediated by TGF-β. Nature (Lond) 371: 257–261

Hinds PS, Mittnacht S, Dulic V, Arnold A, Reed SI and Weinberg RA (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell 70: 993–1006

Huang DP, Ho JHC, Poon YF, Chew EC, Saw D, Liu M, Li CL, Mak LS, Lai SH and Lau EH (1980) Establishment of a cell line (NPC/HK1) from a differentiated squamous carcinomas of the nasopharynges. Int J Cancer 26: 127–132

Huang DP, Lo KW, Choi PHK, Ng AYT, Tsao SY, Yiu GKC and Lee JC (1991) Loss of heterozygosity on the short arm of chromosome 3 in nasopharyngeal carcinoma. Cancer Genet Cytogenet 54: 91–99

Huang DP, Lo KW, van Hasselt CA, Woo JK, Choi PH, Leung SF, Cheung ST, Cairns O, Sidransky D and Lee JC (1994) A region of homozygous deletion on chromosome 9p21–22 in primary nasopharyngeal carcinoma. Cancer Res 54: 4003–4006

Hui ABY, Lo KW, Leung SF, Choi PH, Fong Y, Lee JC and Huang DP (1996) Loss of heterozygosity on the long arm of chromosome 11 in nasopharyngeal carcinoma. Cancer Res 56: 3225–3229

Jin X, Nguyen D, Zhang WW, Kyritsis AP and Rod JA (1995) Cell cycle arrest and inhibition of tumour cell proliferation by the p16[QD] gene mediated by an adenovirus vector. Cancer Res 55: 3250–3253

Kamb A, Graus A, Weaver-Feldhaus J, Liu QY, Harshman K, Tavaglione SV, Stockert E, Day III RS, Johnson BE and Skolnick MH (1994) A cell cycle regulator potentially involved in genesis of many tumor types. Science 264: 436–439

Kato JY, Matsushime H, Hiebert SW, Ewen ME and Sherr CJ (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRB) and pRB phosphorylation by the cyclin D-dependent kinase CDK4. Gene Dev 7: 331–342

Li JH, Li P, Klamut H and Liu FF (1997) Cytotoxic effects of ad5CMV-p53 expression in two human nasopharyngeal carcinoma cell lines. Clin Cancer Res 3: 507–514

Liggett WH Jr, Sewell DA, Rocco J, Ahrendt SA, Koch W and Sidransky D (1996) p16 and p16 are potent growth suppressors of head and neck squamous carcinoma cells in vitro. Cancer Res 56: 4119–4123

Lo KW, Huang DP and Lau KM (1995) p16 gene alterations in nasopharyngeal carcinoma. Cancer Res 55: 2039–2043

Lo KW, Cheung ST, Leung SF, van Hasselt A, Tsang YS, Mak KF, Chung YF, Woo JKS, Lee JCK and Huang DP (1996) Hypermethylation of the p16 gene in nasopharyngeal carcinoma. Cancer Res 56: 2721–2725

Lo KW, Huang DP and Lee JCK (1997) Genetic changes in nasopharyngeal carcinoma (NPC). Chinese Med J (Beijing) 110: 548–559

Lu Qi, Elia G, Lucas S and Thoma IA (1993) Bcl-2 proto-oncogene expression in Epstein-Barr-virus-associated nasopharyngeal carcinoma. Int J Cancer 53: 29–35

Miyakoshi J, Kitagawa K, Yamagishi N, Ohitsu S, Day RS III and Takebe H (1997) Increased radiosensitivity of p16 gene-deleted human glioma cells after transfection with wild-type p16 gene. Jpn J Cancer Res 88: 34–38

Nobori T, Miura K, Wu DJ, Lois A, Takabaryashi K and Carson DA (1994) Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature (Lond) 368: 753–756

Pagano M, Pepper R, Lukas J, Baldwin V, Ansorge W, Bartek J and Draetta G (1993) Regulation of the cell cycle by the cd2kd protein kinase in cultured human fibroblasts. J Cell Biol 121: 110–111

Porter MJ, Field JK, Leung SF, Lo D, Lee JC, Spandidos DA and van Hasselt CA (1996) The detection of the c-myc and ras oncogenes in nasopharyngeal carcinoma by immunohistochemistry. Acta Otolaryngol 114: 105–109

Quesnel B, Preudhomme C, Lepelley P, Hetuin D, Vanrumbeke M, Bauters F, Velu T and Hubert S (1998) Regulation of retinoblastoma protein functions by ectopic expression of human/mouse p19, a novel cdk4/cdk6 inhibitor with homology to p16ink4. Mol Cell Biol 15: 2682–2688

Sugden DB, Suppression of growth in vitro and tumorigenicity in vivo of human nasopharyngeal carcinoma cells transfected with p16[QD]. Mol Carcinogen 16: 53–60

Spruck CH III, Tsai YC, Huang DP, Yang AS, Rideout III WM, Gonzalez-Zulueta M, Choi P, Lo KW, Yu MC and Jones PA (1992) Absence of p53 gene mutations in primary nasopharyngeal carcinomas. Cancer Res 52: 4787–4790