JKGUI 2003; 10 (Edisi Khusus):510-519
Diterbitkan di Jakarta

Jurnal Kedokteran Gigi
Universitas Indonesia
ISSN 0854-364X

TUMOR SUPPRESSINGFUNCTIONS OF $p16^{INK4a}$ – A REVIEW

Auerkari EI*,Suwelo IS*, Tjarta A, Cornain S**, Rahardjo TW*, Eto K***, Ikeda-MA*****

*Faculty of Dentistry-University of Indonesia
**Faculty of Medicine University of Indonesia
***Faculty of Dentistry-Tokyo Medical & Dental University

Auerkari EI, Suwelo IS, Tjarta A, Cornain S, Rahardjo TW, Eto K, Ikeda-MA: Tumor Suppressing Functions of p16INK4a-A review. Jurnal Kedokteran gigi universitas indonesia. 2003; 10 (Edisi Khusus):510-519

Abstract

DNA damage can have particularly severe carcinogenic influence if it incapacitates the cellular machinery normally protecting the cell from the effects of genomic damage. The protective functions involve not only DNA repair and apoptosis (programmed cell death), but also regulation of the cell cycle and proliferation. Therefore, carcinogenesis can be promoted by inactivating or altering key regulatory proteins like $p16^{INK4a}$, which has the capability to arrest the cell cycle in the G_1 phase and prevent inappropriate proliferation. Functional cyclin-dependent kinase (Cdk) inhibitor $p16^{INK4a}$ binds to Cdk-4 and Cdk-6, thereby preventing the Cdk-cyclin complexes from promoting phosphorylation of pRb and releasing the transcription factor E2F, needed for the cell cycle to proceed to the S phase. Arrest in G_1 accounts for a minority of arresting cells after DNA damage, the majority of arrests taking place in G_2 without recognized $p16^{INK4a}$ contribution. However, inactivating alterations of $p16^{INK4a}$ are common in cancers, possibly because of additional functions of $p16^{INK4a}$ in senescence and inhibition of the spreading and migration of cancer cells. Since oncogenic initiation is insufficient for growing significant tumors without spreading and angiogenesis, this could partly explain why inactivated $p16^{INK4a}$ is frequently exhibited in clinical tumors in spite of apparently less exclusive role in cell cycle arrest. On the other hand, multiple oncogenic events are usually necessary to develop cancer, and generally both pRb and p53 pathways are impaired in tumors. This suggests that growth regulation in G_1 and therefore also its key molecular components including $p16^{INK4a}$ are important in carcinogenesis.

Key words: Tumor; DNA

Introduction

In general, carcinogenesis results from a failure of the cellular defences to DNA damage or oncogene activation. This may happen due to some specific weakness in the defence mechanisms, or by chance with sufficient time to accumulate damage.

Cell cycle arrest

DNA damage is particularly severe if it can inactivate the mechanisms that are normally protecting the cell from genomic damage. The protective functions include several mechanisms of DNA repair, apoptosis or programmed cell death in case of irrepairable damage, and arresting of the cell cycle before undue proliferation. Arresting of the cell cycle is also important to allow for the necessary time for DNA repair. Therefore, carcinogenesis can be promoted by inactivating or altering key regulatory proteins like $p16^{INK4a}$, which can mediate arrest of the cell cycle in the G_1 phase.

The progression of the cell cycle is controlled normally by partly interconnected and tightly regulated processes, mediated by a network of promoter and inhibitor proteins (Fig 1). An important part of this cellular machinery is the pRb/E2F pathway, which is involved in the cell cycle regulation at the G_1/S boundary both in stressed and unstressed cells.[1] Active dephosphorylated pRb inhibits the G_1/S transition and cell proliferation by binding the E2F transcription factors. Full phosphorylation of pRb will release E2F and promote progression to the S phase. The phosphorylation is promoted by complexes of cyclin-dependent kinases Cdk 4/6 and Cyclin D, and inhibited by cyclin-dependent kinase inhibitors (Cdi) like $p16^{INK4a}$, which prevent the complex formation by binding themselves to the Cdk, arresting the cell cycle at G_1.[2,3,4,5] If an alteration in the Cdi prevents its inhibitory function, the cell cycle can proceed to proliferation, and in case of sufficient number of oncogenic events, possibly eventually initiating cancer.[7,8,9,10] In the pRb/E2F pathway, pRb binds to E2F1, E2F2, E2F3 and E2F4, and parallel to this, the pRb family proteins p130 and p107 bind to E2F4 or E2F5.[1,2,6] For $p16^{INK4a}$ dependent arrest in G_1, pRb function alone is not sufficient without the action of either p130 or p107.[1] Non-functional $p16^{INK4a}$ can appear in cancer cells regardless of the status of p53, which controls much of the cellular defences after DNA damage.[2,3,4]

The p16 protein and other INK family proteins contain four or five tandemly repeated ankyrin motifs, each about 32 amino acids in length.[10,11,12] The functional binding locus of $p16^{INK4a}$ appears to reside by the third ankyrin repeat, extending somewhat beyond it, and binding adjacent to the ATP-binding site of the Cdk catalytic cleft. This prevents binding of ATP and induces structural changes that propagate to the cyclin-binding site of Cdk.[19,20,21]

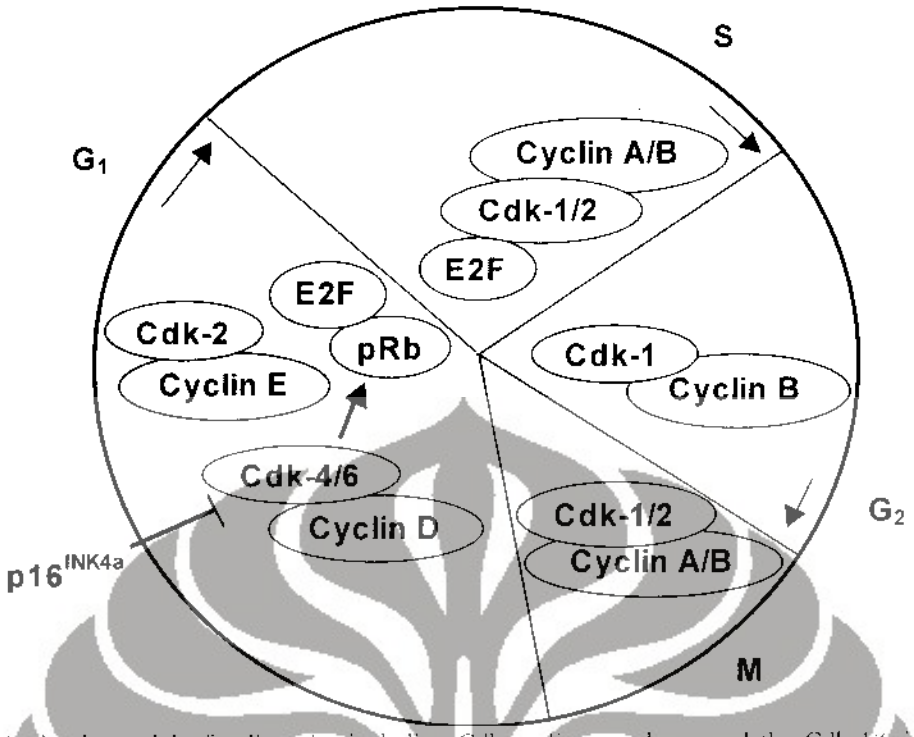

Fig 1. A simple model of cell cycle, including Cdk-cyclin complexes and the Cdk-4,6 inhibitor $p16^{INK4a}$ which can mediate cycle arrest at G_1. With inhibitor action, the Cdk-cyclin complexes cannot promote phosphorylation of the pRb family of proteins to release the E2F transcription factors for G_1/S transition. Note that there are other pathways to G_1 arrest (see Fig 2), and that parallel processes can also arrest G_2 before entering mitosis (M).

There are several other important Cdi proteins (Fig 2) inducing G_1 arrest, like $p21^{Waf1/Cip1}$, which is activated in p53 dependent and independent manner after DNA damage or oncogene activation, and $p27^{Kip1}$, which can bind to and inhibit Cdk-2.[3,4,5,6,15] In spite of the potential complementary action of at least three Cdi molecules, G_1 arrest accounts only for 15-40% of arrested cells after DNA damage.[1,2] A second set of defence mechanisms can arrest the cell cycle in the G_2 phase, preventing the cell from initiating mitosis. Blocking G_2 accounts for 60-85% of the arrested cells after DNA damage, and is largely mediated by p53 dependent regulation without known influence by $p16^{INK4a}$.[2,15]

Studies on tumors with inactive $p16^{INK4a}$ have attributed the inactivation to allelic loss (homozygous deletion) and to transcriptional silencing by subtle mutations or DNA methylation.[11,12,13,16,17,23] DNA methylation occurs in about one third of all samples, particularly affecting the 5' GpG island of the gene. Silencing by methylation may prevent transactivation of *$p16^{INK4a}$* by AP-2, a DNA-binding protein implicated in signaling terminal differentiation and in transactivating also $p21^{Waf1}$.[14,18]

Apart from the assumed transactivation by AP-2, at least the *ras* family of genes, expressed as four 21 kD proteins H-, N-, KA and KB-ras, appear to induce the Cdi proteins $p16^{INK4a}$ and $p21^{Waf1}$.[14,22,23] Oncogenic changes cooperating with *ras* tend to neutralise the functionality of these kinase inhibitors, and can also suppress the third important kinase inhibitor $p27^{Kip1}$ via the MAP kinase pathway.[14,23]

Some of the $p16^{INK4a}$ and pRb related regulatory proteins of the cell cycle and their chromosomal mapping are summarised in Table 1.

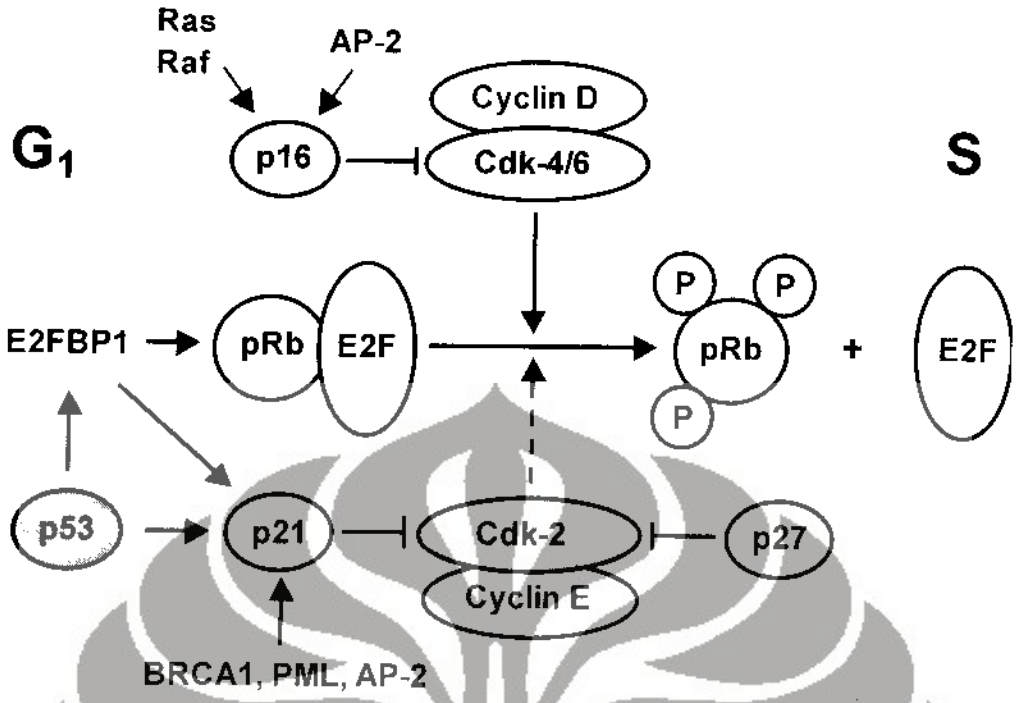

Fig 2. Simple model of the regulatory network for cell cycle arrest in G_1 by cyclin-dependent kinase inhibitors (Cdi) $p16^{INK4a}$, $p21^{Waf1/Cip1}$ or $p27^{Kip1}$. An arrow signifies activation/promotion or process direction, a line with a terminal bar inhibition. Any of the three shown Cdi's can arrest the cycle, since the action of both Cdk-cyclin complexes are needed for passage from G_1 to S. Several of the indicated regulatory molecules appear in reality as a family of proteins with partly parallel functions, e.g. the INK family of kinase inhibitors includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. Also, to arrest G_1 in a $p16^{INK4a}$ dependent manner, parallel function by other members of the pRb family (p130 or p107, not shown) is also needed in addition to pRb.

The three kinase inhibitors $p16^{INK4a}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ generally respond to different sets of stimuli and are not of equal importance under particular circumstances to arrest the cellular cycle. The Cdi's also show unequal rates of carcinogenic alterations. In observed cases, $p16^{INK4a}$ has been found frequently mutated or otherwise altered in tumors, but $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ much less often.[14] Tumorigenesis is thought to require on average about six oncogenic events[7,8], but these events can be both complementary and exclusive. For example, silencing of $p16^{INK4a}$ in tumors is usually accompanied with alteration of some of the central molecules of the p53 pathway (such as p53 itself), demonstrating the powerful carcinogenic effect of combined inactivation of p53 and pRb pathways. Within the pRb pathway either $p16^{INK4a}$ or pRb (or other pRb family proteins p130 or p107) can be altered, but generally not both, i.e. expression and alteration of these components are inversely correlated.[25,26,28,29]

Table 1. Some important regulatory components in the cell cycle and defence systems against DNA damage, with main functions and chromosomal locations.

Gene/expression	Main function	Chromosomal mapping
p53	Damage checkpoint, arrest/apoptosis	17p13.1
$p14^{ARF}$	Binds MDM2 to stabilise p53 and arrest G_1	9p21-22 [1]
$p16^{INK4a}$	Induces G_1 arrest mediated by pRb, p130, p107	9p21-22 [2]
$p21^{Waf1}$	Inhibits Cdk-cyclin, arrest of G_1	6p21.2
$p27^{Kip1}$	Inhibits Cdk-2, arrest of G_1	12p12 - 12p13.1
pRb	Retards G_1 to S transition with E2F	13q14.2
Cyclin D	Control of G_1 to S transition	11q13

1) reading exons 1β, 2 and 3; 2) alternative reading exons 1α, 2 and 3

Inhibition of cell migration

The $p16^{INK4a}$ protein is a small molecule and its function as a G_1 kinase inhibitor can be mimicked by a 10 amino acid peptide, which corresponds to the third ankyrin-like repeat of the full-length protein.[10] This peptide can be synthesized and directly delivered to cells, with 100 % efficiency in arresting the cells reversibly at G_1. However, the peptide also shows matrix-specific inhibition of cells spreading on vibronectin, without affecting the capacity of the cells to attach to this substratum. This effect is related to dissociation of the vitronectin intergrin receptor αvβ3 from the focal adhesion contacts. Similar effect has been observed using peptides derived from the kinase inhibitor $p21^{Waf1/Cip1}$, suggesting that the G_1 associated kinase inhibitors regulate αvβ3 function.[19] It has also been found that transfection of glioblastoma and melanoma cells with full length *$p16^{INK4a}$* gene construct results in inhibition of spreading and cell migration. As αvβ3 is strongly associated with invasive cancer and angiogenesis, it is possible that $p16^{INK4a}$ (and some other G_1 kinase inhibitors) can inhibit tumor growth as well as initiation.[10]

The G_1 arrest accounts only for a minority, about 15-40% of arresting cells, the majority of arrests taking place in the G_2 phase where $p16^{INK4a}$ has no appreciable influence.[2,14] However, mutations or other inactivating alterations of $p16^{INK4a}$ are relatively common in cancers. One possible reason for this could be the suggested additional role of $p16^{INK4a}$ in inhibiting the spreading and migration of cancer cells, and possibly even angiogenesis.[10,24] Particularly solid tumors cannot grow beyond a size of about 1-2 mm without additional new blood vessels.[9] Although not applicable to all cancer types, initiation of such a small stagnant tumor is usually insufficient to develop a clinically significant disease without spreading and angiogenesis. This could partly explain why inactivated $p16^{INK4a}$ is frequently exhibited in clinical tumors, as clinically significant large and growing tumors are much more likely to be

detected than small stagnant tumors. There appears to be little comparative evidence on alterations of $p16^{INK4a}$ in small stagnant and larger growing tumors. General comparisons of premalignant and malignant tumors[29] do not provide such evidence, since most studies on premalignant tissue have not included follow-up on stagnancy. However, absence of $p16^{INK4a}$ tends to correlate with reduced survival, while e.g. loss of pRb expression does not, in spite of observed inverse correlation between loss of pRb and $p16^{INK4a}$ expressions[1,25] This suggests influence by other functions of $p16^{INK4a}$ than cell cycle arrest on the pRb pathway.

Control of cellular proliferative life span

Normal somatic cells have a limited life span in terms of the number of possible cell divisions. This limit is generally attributed to gradual shortening of telomeres until they are too short to sustain further cell divisions. However, loss of function of $p16^{INK4a}$ or p53 can considerably extend the limit, and in this extension the effect of these two proteins is additive.[1,30] Eventually such cells stop dividing and reach a state called crisis. Small number of cells may escape the crisis limit and become immortalised by activating telomere maintenance, usually with the telomerase enzyme. In this process loss of $p16^{INK4a}$ hence appears to occur before immortalisation.[14]

Immortalisation is a common though not exclusive feature of tumor cells. Frequent loss of $p16^{INK4a}$ has been observed in immortalised tumor cell lines, while also several immortalised but not tumorigenic *in vitro* cell lines have been found to lack functional $p16^{INK4a}$.[10,11,14] Induced expression of $p16^{INK4a}$ (or $p21^{Waf1/Cip1}$) in immortalised human fibroblasts leads to senescence-like growth arrest.[31] Spontaneous loss of $p16^{INK4a}$ expression has been seen in small colonies of normal human mammary epithelial cells grown on standard serum-free medium, resulting in additional 40-50 divisions beyond the normal limit, until final permanent growth arrest.[32,33] Such spontaneous loss of of $p16^{INK4a}$ expression has been attributed to methylation of the CpG island of the gene, and treatment of the cells with the methylation inhibitor 5-aza-2'-deoxycytidine has been shown to restore $p16^{INK4a}$ expression with the accompanying normal growth arrest.[34]

In certain cells like normal fibroblasts and retinal pigment epithelial cells, transduction of the telomerase catalytic subunit (hTERT) cDNA is sufficient for a large extension of the life span.[35,36] However, other cell types like keratinocytes or human mammary epithelial cells only show this extension if either pRb or $p16^{INK4a}$ is inactivated. The cell type dependent differences of the extension appear not to be well understood.[14,37]

In several human cell types the levels of $p16^{INK4a}$ tend to accumulate, so that a senescent cell may contain up to 40 times the levels observed in early passage cells.[38] The continuous accumulation with the number of cell divisions suggests that the increase in $p16^{INK4a}$ levels is not a consequence of reaching senescence, and it has been speculated that accumulation of $p16^{INK4a}$ could be involved in triggering senescence or possibly related to progressive telomere shortening or other mitotic clock mechanisms.[14]

Expression of *ras* or *raf* oncogenes can induce accumulation of $p16^{INK4a}$ (and p53), and premature senescence.[22,23] This function of induction of premature senescence has also been found from other cyclin-dependent kinase inhibitors.[39] The kinase inhibitors therefore show partially shared complementary functions to provide redundance for the cellular defences. These functions link the mechanisms of senescence to those of cell cycle arrest.

Discussion

High reactivity of the human genome can result in more than 100 modifications a day.[7,8] This is significant attack in spite of the large size of the genome, also because the modifications and

their consequences are not random. They inflict particularly severe carcinogenic consequences if they can disable some of the key mechanisms in the cellular defences to damage or inappropriate oncogene activation. Therefore, the observed alterations in tumors concentrate on key regulatory proteins like $p16^{INK4a}$ and other tumor suppressors in the defence networks.[1,2,12] Alteration or loss of $p16^{INK4a}$ has been often implicated in a wide range of different types of cancers, including head and neck, esophageal, lung, brain, breast, colon, bladder and bone cancers as well as gliomas, T-cell acute lymphoblastic leukemias and melanomas.[16,17,18] In general, albeit insufficient to initiate cancer alone, defective $p16^{INK4a}$ is the most common type genomic damage in human cancer after defective p53, and frequently correlated with reduced survival.[4,23,28]

Understanding of the functions of $p16^{INK4a}$ has been complicated by the unusual character of the corresponding gene locus. Through alternative reading frames this locus encodes two distinctly different regulatory proteins, $p16^{INK4a}$ and $p14^{ARF}$, of which the latter induces p53-dependent growth arrest by binding MDM2, stabilising p53 and thereby promoting $p21^{Waf1\ Cip1}$ to inhibit kinase-cyclin complexes required for G_1/S transition.[2,14] Genomic alteration affecting $p16^{INK4a}$ can also have an impact on $p14^{ARF}$, and therefore without checking it is possible that phenomena due to alterations in both proteins are incorrectly assigned to only one of them. There is evidence suggesting that the expression of both of these proteins is regulated by a common transcriptional repressor bmi-1[15], directly linking the growth arrest functions of the pRb and p53 pathways.

G_1 arrest accounts only for a minority, about 15-40% of arresting cells after DNA damage, the majority of arrests taking place in G_2 where $p16^{INK4a}$ has no known influence[2,14], and yet mutations or other inactivating alterations of $p16^{INK4a}$ are relatively common in cancers. A possible explanation can be found in the suggested functions of $p16^{INK4a}$ in inhibiting the spreading and migration of cancer cells, and possibly even angiogenesis.[12] Solid tumors are believed to have a maximum size of about 1-2 mm without angiogenesis or additional blood vessels to maintain growth. Initiation of a single tumor would therefore not lead to a clinically significant disease without spreading and angiogenesis. In addition, loss of $p16^{INK4a}$ function may facilitate avoiding cellular senescence, which is also an important defence mechanism against genomic damage and oncogenesis. The roles in tumor growth and senescence could partly explain why dysfunctional $p16^{INK4a}$ is frequently exhibited in clinical tumors.

On the other hand, a chain of about six oncogenic events are believed to be necessary to develop cancer, and within this chain generally both the pRb and p53 pathways are impaired in tumors.[7,8] This alone suggests that growth regulation in G_1 is significant in carcinogenesis, and therefore potentially also the functional role of $p16^{INK4a}$ as a cyclin-dependent kinase inhibitor. Within this explanation, the importance of $p16^{INK4a}$ is emphasised by the observation that alterations of $p16^{INK4a}$ and pRb are inversely correlated in tumors.[25,26,29] This suggests that the tumor suppressor function of $p16^{INK4a}$ has considerable uniqueness in spite of existing other members of the INK family of proteins, so that $p16^{INK4a}$ exhibits limited redundance within the pRb pathway. Although there are other cyclin-dependent kinase inhibitors of both p53 dependent and independent pathways, such as $p21^{Waf1\ Cip1}$ and $p27^{Kip1}$, these do not share same routes of promotion or impact with $p16^{INK4a}$. Also, little redundance to $p16^{INK4a}$ or pRb functions is provided by p130 and p107, which have been also found to be altered in some tumors.[6] Not much redundance can be expected from $p14^{ARF}$ either, since due to shared coding locus both protein expressions can be dysfunctional when $p16^{INK4a}$ is altered.

The performance and redundancies in the network of cellular defences are characterised by the number of oncogenic

events required for carcinogenesis, as well as the relative difficulty by the oncogenic agents to induce them. Individual molecular components of the defences are much less redundant than the system, and with unequal oncogenic weight. Therefore molecules like $p16^{INK4a}$ with multiple important functions are often dysfunctional in tumors. For reversing the processes that support tumor initiation and growth, it may hence suffice to interfere with the dysfunction of very few key molecules within the cellular defences. This is also an essential starting point for much of the current effort towards new therapeutic drugs.

References

1. Bruce, J.L., Hurford, R.K.Jr., Classon, M., Koh, J. & Dyson, N., Requirements for cell cycle arrest by $p16^{INK4a}$. *Molecular Cell* 2000. 6: 737-742.
2. Scully, C., Field, J.K. & Tanzawa, H., 2000. Genetic aberrations in oral or head and neck squamous cell carcinoma (SCCHN): 1. Carcinogen metabolism, DNA repair and cell cycle control. *Oral Oncology* 2000. 36: 256-263.
3. Gartel, A.L. & Tyner, A.L., Transcriptional regulation of the $p21^{(WAF1/CIP1)}$ gene. *Experimental Cell Research* 1999. 246: 280-289.
4. El-Deiry, W.S., Regulation of p53 downstream genes. *Cancer Biology*1998. 8: 345-357.
5. Ko, L.J. & Prives, C., p53: puzzle and paradigm. *Genes and Development* 1996. 10: 1054-1072.
6. Vairo, G., Soos, T.J., Upton, T.M., Zalvide, J., DiCaprio, J.A., Ewen, M.E., Koff, A. & Adams, J.M., Bcl-2 retards cell cycle entry through $p27^{kip1}$, pRB relative p130, and altered E2F regulation. *Molecular and Cellular Biology* 2001. 20(13): 4745-4753.
7. Renan, M.J., How many mutations are required for tumorigenesis? Implications from human cancer data. Molecular Carcinogenesis 1993. 17: 139-146.
8. Steen, H.B., The origin of oncogenic mutations: where is the primary damage? *Carcinogenesis*2000. 21(10): 1773-1776.
9. Macluskey, M., Chandrachud, L.M., Pazouki, S., Green, M., Chisholm, D.M., Ogden, G.R., Schor, S.L. & Schor, A.M., Apoptosis, proliferation and angiogenesis in oral tissues. Possible relevance to tumour progression. *Journal of Pathology* 2000. 191: 368-375.
10. Fahraeus, R., New approaches to cancer therapies. *Journal of Pathology* 1999. 187: 138-146.
11. Loughran, O., Malliri, A., Owens, D., Gallimore, P.H., Stanley, M.A., Ozanne, B., Frame, M.C. & Parkinson, Association of CDKN2A/p16 INK4A with human head and neck keratinocyte replicative senescence: relationship of dysfunction to immortality and neoplasia. *Oncogene*1996. 13: 561-568.
12. Cody, D.T., Huang, Y., Darby, C.J., Johnson, G.K. & Domann, F.E., Differential DNA methylation of the *p16 INK4A/CDKN2A* promoter in human oral cancer cells and normal human oral keratinocytes. *Oral Oncology* 1999. 35: 516-522.
13. Paterson, I.C., Eveson, J.W. & Prime, S.S., Molecular changes in oral cancer may reflect aetiology and ethnic origin. *Oral Oncology* 1996. 32B(3):150-153.
14. Huschtscha, L.I. & Reddel, R.R., $p16^{INK4a}$ and the control of the cellular proliferative life span. *Carcinogenesis* 1999. 20(6):921-926.
15. Jacobs, J.J.L., Kieboom, K., Marino, S., DePinho, R.A. & van Lohuizen, M., The oncogene and Polycomb-group gene *bmp-1* regulates cell proliferation and senescence through the *ink4a* locus. Nature 1999. 397: 164-168.
16. Liggett, W.H. Jr. & Sidransky, D., Role of the *p16* tumor suppressor gene. *Journal of Clinical Oncology* 1998. 16: 1197-1206.
17. Herman, J.G., Merlo, A., Mao, L., Lapidus, R.G., Issa, J.P.J., Davidson, N.E., Sidransky, D. & Baylin, S.B., Inactivation of the *CDKN2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Research* 1995.55: 4525-4530.
18. Shapiro, G.I., Edwards, C.D., Kobzik, L., Godleski, J., Richards, W., Sugarbaker, W.J. & Rollins, B.J., 1995. Reciprocal Rb inactivation and $p16^{INK4}$ expression in primary lung cancers and cell lines. *Cancer Research* 1995.55: 505-509.
19. Fahraeus, R., Lain, S., Ball, K. & Lane, D.P., Characterisation of the cyclin-dependent kinase inhibitory domain of the INK4 family as a model for a synthetic tumour suppressor molecule. *Oncogene*1996. 16:587-596.

20. Russo, A.A., Tong, L., Lee, J.-O., Jeffrey, P.D. & Pavletich, N.P., Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumor suppressor $p16^{INK4a}$. *Nature* 1998. 395: 237-243.
21. Brotherton, D.H., Dhananaj, V., Wick, S., Brizuela, L., Domaille, P.J., Volyanik, E., Xu, X., Parisini, E., Smith, B.O., Archer, S.J., Serrano, M., Brenner, S.L., Blundell, T.L. & Laue, E.D., Crystal structure of of the complex of the cyclin D dependent kinase Cdk6 bound to the cell-cycle inhibitor $p19^{INK4d}$. *Nature* 1998. 395: 244-250.
22. Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. & Lowe, S.W., *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16*. Cell 1997. 88: 593-602.
23. Lin, A.W., Barradas, M., Stone, J.C., Van Aelst, L., Serrano, M. & Lowe, S.W., Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev* 1998. 12:. 3008-3019.
24. Claudio, P.P., Stiegler, P., Howard, C.M., Bellan, C., Minimo, C., Tosi, G.M., Rak, J., Kovatich, A., De Fazio, P., Micheli, P., Caputi, M., Leoncini, L., Kerbel, L., Giordano, G.G. & Giordano, A., RB2/p130 gene-enhanced expression down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in vivo. *Cancer Research* 2001. 61(2):462-468.
25. Maitra, A., Roberts, H., Weinberg, A.G. & Geradts, J., 2001. *Loss of p16 (INK4a) expression correlates with decreased survival in pediatric* osteosarcomas. University of Texas, Southwest Medical Center, USA.
26. Nakahara, Y., Shintani, S., Mihara, M., Kiyota, A., Ueyama, Y. & Matsumura, T., Alterations of Rb, $p16^{INK4A}$ and cyclin D1 in the tumorigenesis of oral squamous cell carcinomas. *Cancer Letters* 2000. 160: 3-8.
27. Akanuma, D., Uzawa, N., Yoshida, M.A., Negishi, A., Amagasa, T. & Ikeuchi, T., Inactivation patterns of the *p16 (INK4a)* gene in oral squamous cell carcinoma cell lines. *Oral Oncology* 1999. 35: 476-483.
28. Lin, S-C., Chang, K-W., Chang, C-S., Liu, T-Y., Tzeng, Y-S., Yang, F-S. & Wong, Y-K., Alterations of p16/MTS1 gene in oral squamous cell carcinomas from Taiwanese. *Journal of Oral Pathology and Medicine* 2000. 29: 159-166.
29. Pande, P., Mathur, M., Shukla, N.K. & Ralhan, R., pRb and p16 protein alterations in human oral tumorigenesis. *Oral Oncology* 1998. 34: 396-403.
30. Noble, J.R., Rogan, E.M., Neumann, A.A., Maclean, K., Bryan, T.M. & Reddel, R.R., Association of extended in vitro proliferative potential with loss of $p16^{INK4}$ expression. *Oncogene*1996. 13: 1259-1268.
31. Vogt, M., Haggblom, C., Yeargin, J., Christiansen-Weber, T. & Haas, M., Independent induction of senescence by $p16^{INK4a}$ and $p21^{CIP1}$ in spontaneously immortalized human fibroblasts. *Cell Growth and Differentiation* 1998. 9: 139-146.
32. Brenner, A.J., Stampfer, M.R. & Aldaz, C.M., Increased *p16* expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with *p16* inactivation. *Oncogene*1998. 17: 199-205.
33. Huschtscha, L.I., Noble, J.R., Neumann, A.A., Moy, E.L., Barry, P., Melki, J.R., Clark, S.J. & Reddel, R.R., Loss of $p16^{INK4}$ expression by methylation is associated with lifespan extension of human mammary epithelial cells. *Cancer Research*1998. 58: 3508-3512.
34. Foster, S. A., Wong, D.J., Barrett, M.T. & Galloway, D.A., Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Molecular Cell Biology* 1998. 18; 1793-1801.
35. Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C-P., Morin, G.B., Harley, CB., Shay, J.W., Lichtsteiner, S. & Wright, W.E., Extension of life-span by introduction of telomerase into normal human cells. *Science*1998. 279 : 349-352.
36. Morales, C.P., Holt, S.E., Ouellette, M., Kaur, K.J., Yan, Y., Wilson, K.S., White, M.A., Wright, W.E. & Shay, J.W., Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nature Genetics* 1999. 21; 115-118.
37. Kiyono, T., Foster, S.A., Koop, J.I., McDougall, J.K., Galloway, D.A. & Klingelhutz, A.J., Both $Rb/p16^{INK4a}$ inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* 1998. 396: 84-88.
38. Alcorta, D.A., Xiong, Y., Phelps, D., Hannon, G., Beach, D. & Barrett, J.C., Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proceedings of the National Academy of Science*, USA. 1996. 93: 13742-13747.

39. McConnell, B.B., Starborg, M., Brookes, S., & Peters, G., Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. *Current Biology*1998. 8: 351-354.