

Molecular basis of cancer

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Cancer, for the most part, is caused by multiple somatic mutations in a single cell and its progeny. However, in some individuals, constitutional genetic alterations may also play a role. Depending on the specific cell type, the affected cell and its progeny accumulate sequential mutations and sustain multiple genetic alterations over decades. The defective genetic anomalies lead to disabled critical cellular pathways, which with DNA replications in between, evolve clonally and expand into a malignant phenotype. Additional mutations in some genes confer a further selective growth advantage and the neoplastic process progresses to invade surrounding tissues and metastasise to other organs.

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Introduction

In this paper, the concepts of oncogenesis are discussed with special emphasis on Knudson's hypothesis,¹ the multistep model, and clonal evolution. Recent advances in this field are also highlighted, with a view to giving an overall picture of the molecular basis of cancer. These are illustrated with examples from our local experience in nasopharyngeal carcinoma (NPC) and multiple myeloma (MM).

Knudson's two-hits model of tumourigenesis

Tumours that occur in children obviously do not take decades to develop. In the tumourigenesis of childhood tumours such as those of the eye and kidney, only two mutations are needed for cancer formation. The two-hits mechanism proposed by Knudson was first confirmed in retinoblastoma.^{1,2} According to Knudson, this cancer occurs as a result of two genetic events in the retinal cell, which result in inactivation of both

copies of a given tumour suppressor, the retinoblastoma gene (*RB*).²

The essential features of Knudson's two-hits model are that in the familial form of cancer, the affected person inherits a mutated allele from one parent (thus having only one copy of the normal gene present in all cells) and a somatic mutation in the target tissue inactivates the normal allele inherited from the other parent. In non-hereditary cancers, both inactivating mutations have to occur within the same somatic cell.² Malignancy is therefore likely to be a more frequent occurrence in those individuals who carry a heterozygous mutation in their germline—i.e. those with the predisposing gene(s) and hence, a predisposition to cancer. Inheritance of the predisposition follows a dominant pattern even though it is transmitted by recessive mutations.

The need for two hits—now known to constitute damage to genes—explains why patients in cancer-prone families are not riddled with tumours throughout their bodies. Inheritance of just one genetic defect predisposes a person to cancer but does not cause it directly as a second event is required.² On the other hand, there is now unequivocal evidence that shows that a certain percentage of human cancers are developed as a consequence of inheriting cancer suscepti-

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bility genes. It is estimated that about 5% to 10% of breast cancer cases may be due to an inherited predisposition.³

The multistep concept

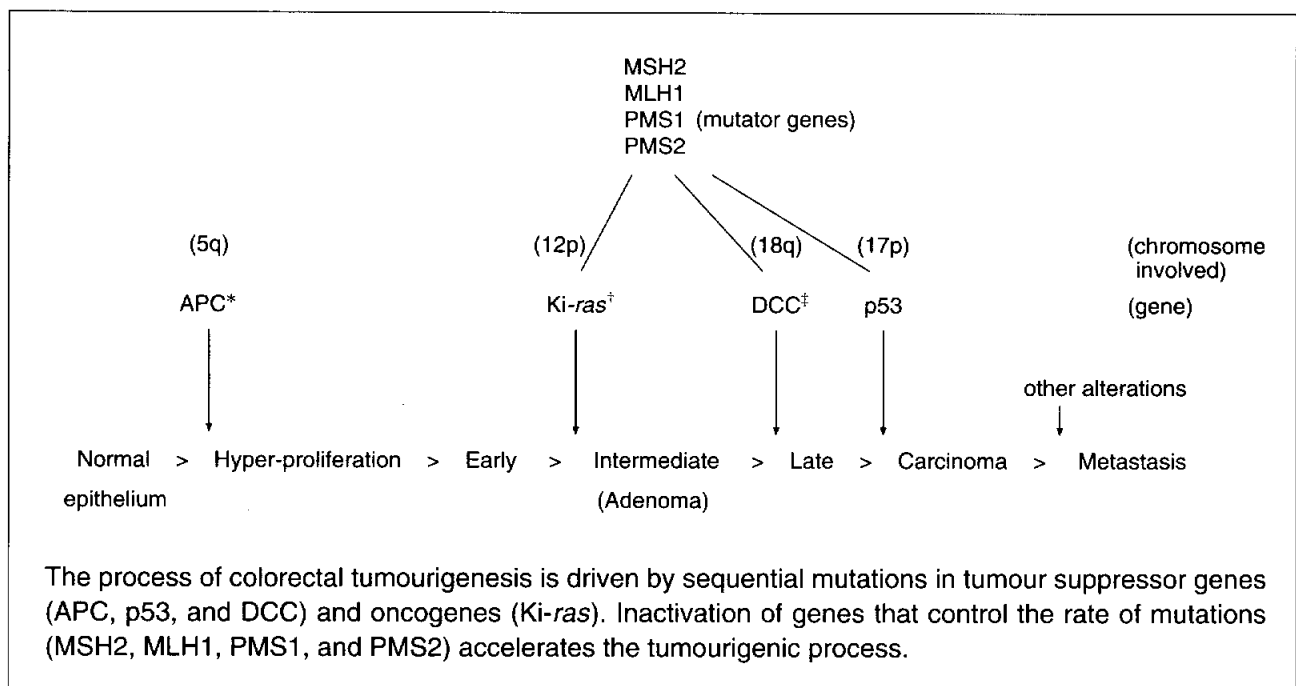
For adult non-haematopoietic solid tumours, the molecular mechanisms responsible for cancer development are much more complex and can be explained by the multistep concept.

It is now generally accepted that most sporadic solid tumours result from a series of clonal expansions and a multistep process of accumulated genetic alterations in cells. For the initiation and progression of disease, the model proposed for colorectal cancer has become a paradigm for other human solid tumours, including brain, bladder, and head and neck cancers.^{4,9} Colorectal cancer provides an excellent example of a human tumour type that can be productively studied. These tumours are prevalent and progress through easily recognisable stages ranging from very small benign polyps (adenomas) to large malignant cancers (carcinomas). Tumour tissue can be easily obtained, examined biochemically, and genetically compared with appropriate control cells from normal colorectal epithelium.

Much of the progress in colon cancer genetics is the result of the selective analysis of kindreds with inherited colon cancer syndromes. The study of familial adenomatous polyposis highlighted an area of chromosomal loss on chromosome 5q.¹⁰ More detailed linkage studies facilitated the precise localisation of the adenomatous polyposis coli (*APC*) tumour suppressor gene.^{11,12} Mutations were then identified in affected family members that typically resulted in a truncated protein product.¹³ When sporadic colon cancers were analysed, similar *APC* mutations were found to be prevalent.¹⁴ The appearance of an inherited mutant allele of *APC* or acquisition of a somatic mutation represents one of the earliest steps that leads to dysplastic lesions of this cancer.^{4,5} Knudson's initial insight that the causes of sporadic and familial cases can involve the same gene and the same biochemical abnormalities (the *RB* gene in retinoblastoma) has been confirmed in other cancers, for example, the *APC* gene for colorectal cancer.

In colon neoplasia, samples from the successive stages were collected and compared for their genetic abnormalities. The molecular profile at each stage has been elucidated⁵ (Fig 1). The *APC* tumour suppressor gene mutations occurred in up to 63% of premalignant

Fig 1. A genetic model for colorectal tumourigenesis⁵



**APC* adenomatous polyposis coli

[†]*Ki-ras* a member of the *ras* gene family, identified as the oncogene of the Kirsten sarcoma viruses

[‡]*DCC* deleted in colorectal cancer

Table. The role of cancer genes in the development of human cancer⁶⁰

	Oncogenes	Tumour suppressor genes	Mismatch repair genes
No. of mutational events needed to contribute to cancer	One (dominant)	Two (recessive)	Two (recessive)
Function of the mutant allele	Gain	Loss	Loss
Origin of the mutation	Somatic	Inherited or somatic	Inherited or somatic
Mechanism of action	Part of the signal transduction pathway	Negative regulation of cell division	Maintain fidelity of DNA replication process

nant adenomatous polyps.¹⁴ As adenomas progress, they accumulate mutations in the oncogene *ras*.¹⁵ Later-stage carcinomas are characterised by the additional loss of regions on chromosomes 18q and 17p, where the tumour suppressor genes *DCC* (deleted in colorectal cancer) and *p53* are localised.⁴ It has been further observed that so long as a sufficient number of critical pathways are disabled, tumour growth ensues. In fact, it is likely that the constellation of genetic alterations is more important than the order in which they are acquired and that this sequence varies in individual patients in the multistage process of cancer development.

Clonal evolution

Both kinds of mutations (oncogene activation and tumour suppressor gene loss) are involved and accumulated in one cell and its direct descendants by a process known as clonal evolution. The inappropriately dividing cells copy their DNA and give identical sets to their offspring. One of these cells or its descendants undergoes a mutation that further enhances its ability to escape normal regulation. Repetition of the process enables one cell to accumulate the mutations it needs to metastasise and colonise other organs. Each tumour cell clone follows its own genetic path as it evolves towards malignancy.⁴

Cancer-related genes

From the colorectal cancer model, it is evident that two major categories of genes may be involved in the

pathogenesis of cancer—the proto-oncogenes and the tumour suppressor genes (Table). Mutations that cause cancer alter the protein products of the genes that regulate entry of the cell into the cell division cycle. Activation of the proto-oncogene (normal) to oncogene (cancer gene) exerts a positive regulatory control. This gain of function is seen as dominant-acting in the sense that the mutated allele is dominant and overrides the normal allele.

By contrast, tumour suppressor genes, which are believed to be involved in the normal suppression of cellular proliferation and exert a negative regulation of the cell cycle, are commonly inactivated in tumours. The loss of function is either due to mutations, deletions, and/or epigenetic changes (DNA methylation).¹⁶ Tumour suppressor gene mutations are recessive to the normal allele thus necessitating the inactivation of the second wild-type allele for tumour formation—following the two-hits mechanism proposed by Knudson. The germline determinants of almost all familial cancers are found to be mutant alleles of tumour suppressor genes. Because there are safeguards built into the system, more than one mutation must occur before a cancer will form.

Genes responsible for the maintenance of genomic integrity: DNA repair defects and cancer susceptibility

Other than the aforementioned two major categories of cancer genes that mutate during tumour progression or are passed in mutant form through the germline

and participate in regulating cell proliferation, there is evidence that individuals who lack specific DNA repair genes are also predisposed to develop cancer.

The hereditary nonpolyposis colorectal cancer (HNPCC) syndrome is a familial colon cancer syndrome that is far more common than familial adenomatous polyposis, accounting for 3% to 6% of all colorectal tumours. The HNPCC tumour-susceptibility gene has been identified and mapped to chromosome 2p and is known to be responsible for repairing mismatched nucleotides in DNA.

When a strand of DNA is being copied, the protein of this gene seems to act as a "spell checker" and corrects errors made in the pairing of bases. Analysis of large cohorts of HNPCC-associated colon cancers shows that nearly 86% of tumours have "microsatellite instability"¹⁷ e.g., the presence of altered or unstable microsatellite repeat numbers in microsatellites scattered throughout the genome. It is estimated that the total number of mutations at microsatellite loci in replication error positive (RER+) tumour cells could be up to 100-fold that in replication error negative (RER-) cells.¹⁸ Microsatellite instability or replication errors in the defective human DNA mismatch repair genes suggests a mutation affecting DNA replication or repair predisposing to replication errors. This has been shown to be involved in the pathogenesis of HNPCC.¹⁹ Analysis of sporadic tumours belonging to the HNPCC spectrum (colorectal cancer, endometrial cancer, and gastric cancer) reveal a significant proportion of cases with multiple replication errors, as in the HNPCC cases.

The DNA mismatch repair gene mutations in HNPCC revealed the relationship between the defective DNA mismatch repair mechanism and the development of cancer. The onset of many common tumours is substantially accelerated by inapparent DNA repair defects that become unmasked only in premalignant tissue during the course of tumour progression. These defective repairs involve genes responsible for the maintenance of genomic integrity, the malfunction of which may contribute to cancer susceptibility by a rapid accumulation of mutations and speeding up the neoplastic process. They are members of a class of cancer-related genes that do not intrinsically control cell growth, but control the rate of mutation of growth-regulating genes—the oncogenes and tumour suppressors. In the genesis of human cancer, a multiple-hit process, it is thought that mutations in DNA repair genes accelerate the rate of mutations and resulting carcinogenesis. Hence, HNPCC may be thought of as a disease of accelerated tumour progression.

Reactivation of telomerase activity in human cancer

In addition to alterations of the cancer-related genes, recent studies suggest that reactivation of telomerase activity may be a necessary event for the sustained growth of almost all human cancers. The ends of chromosomes are specialised nucleoprotein structures called telomeres, which are essential for the stability and maintenance of all chromosomes. When a telomere declines to a threshold level, a signal is emitted that prevents the cell from dividing further. The telomeres shorten progressively with each cell division in normal somatic cells. In contrast to normal cells, tumour cells show no loss of average telomere length with cell division and thus have the ability to expand indefinitely. It is thought that the immortalisation of the cancer cells is due to the reactivation of telomerase activity, which maintains a stable telomere length. Studies demonstrate that telomerase activity is expressed in most human tumour tissues but not in normal tissues, except those of the germline and rare haematopoietic stem cells. Such findings suggest that this enzyme may be an important biological marker in cancer diagnosis and may serve as a good target for anti-cancer drugs.^{20,21}

What role do viruses have in the molecular pathogenesis of cancer?

Oncogenic viruses provide their host cells with additional growth stimuli. Viral oncoproteins interact with cellular regulatory genes; they override growth suppressor signals and deregulate cell-cycle control.²² Some viral oncoproteins interfere with specific cellular signal transduction pathways. Others modulate cellular transcription factors, and result in increased cell proliferation or affect normal cellular differentiation.^{22,23} Different oncogenic viruses target different subsets of the cell-cycle regulatory pathways and/or trans-activate different cellular genes.²²

Cervical cancer, for example, represents one type of human tumour that has a consistent and close association with a specific human virus—the human papilloma virus (HPV). Approximately 85% to 90% of cervical cancers contain HPV DNA sequences. Oncogenes in the genomes of HPV type-16 and type-18 produce proteins called E6 and E7, which have been shown to bind to and inactivate the tumour suppressor gene functions of *RB* and *p53*.^{22,24} The binding of E7 to *RB* is functionally equivalent to the phosphorylation of *pRB* by cyclin-dependent kinase 4 complex (cyclinD-cdk4), and so the need for the usual cellular signal is bypassed. Similarly,

the E6 protein combines with the cellular *p53* protein and promotes its degradation. The normal function of *p53*, both as a cell cycle negative regulator and as guardian of the genome is abolished. The inactivation of the tumour suppressors by viral oncoproteins is thought to increase the chances of a precursor lesion turning into cervical cancer.

The genetic alterations found in multiple myeloma

In multiple myeloma (MM), a plasma cell tumour derived from B-lineage clonogenic cells and both oncogenes and tumour suppressor genes are involved. The proto-oncogenes *c-myc* and *bcl-2*, which enhance cell proliferation and survival are consistently over-expressed in the absence of structural rearrangements.²⁵⁻²⁸ Mutations of *ras* and *p53* have been observed at low frequencies.²⁹ Tumourigenesis of MM also follows a multistep process with evidence of clonal evolution.^{30,31} This is supported by the fact that in some MM cases, *p53* mutations have been observed with disease progression but not at presentation. Recent findings using fluorescence-in-situ-hybridisation reveal a high incidence of hemizygous deletions of the *RB* gene, which functions to suppress cell growth, and the production of IL-6, an important myeloma growth factor.^{32,33} Nevertheless, inactivation of the *RB* gene has not been observed.³⁴ This may suggest that an alternative target gene localised on 13q may be involved in MM tumourigenesis.

However, we recently demonstrated that the cell cycle control through the *RB* gene could be dysregulated in MM, as a result of abnormalities of the up-stream regulators, *p16* and *p15*.³⁵ As cyclin-dependent kinase (CDK4/6) inhibitors, *p16* and *p15* inhibit the CDK interaction with cyclin D1, thus preventing the phosphorylation of the *RB* protein (pRb). A consequence of this is that the cell is arrested at the G1 phase.³⁶ Inactivation of *p16/p15* leads to cellular proliferation by promoting entry into the S-phase of the cell cycle. The regulator *p15* is up-regulated by transforming growth factor- β (TGF- β), which plays an important role in growth suppression of the haematopoietic progenitors in the bone marrow (Fig 2).

Both *p16* and *p15* are commonly inactivated by homozygous deletions. These have been observed in many human neoplasias and also lymphoid malignancies, particularly paediatric acute lymphoblastic leukaemias of B-precursor phenotypes.³⁷⁻³⁹ Despite sharing the B-lineage origin, no deletions have been observed in MM.³⁹ In recent analysis, we demonstrated

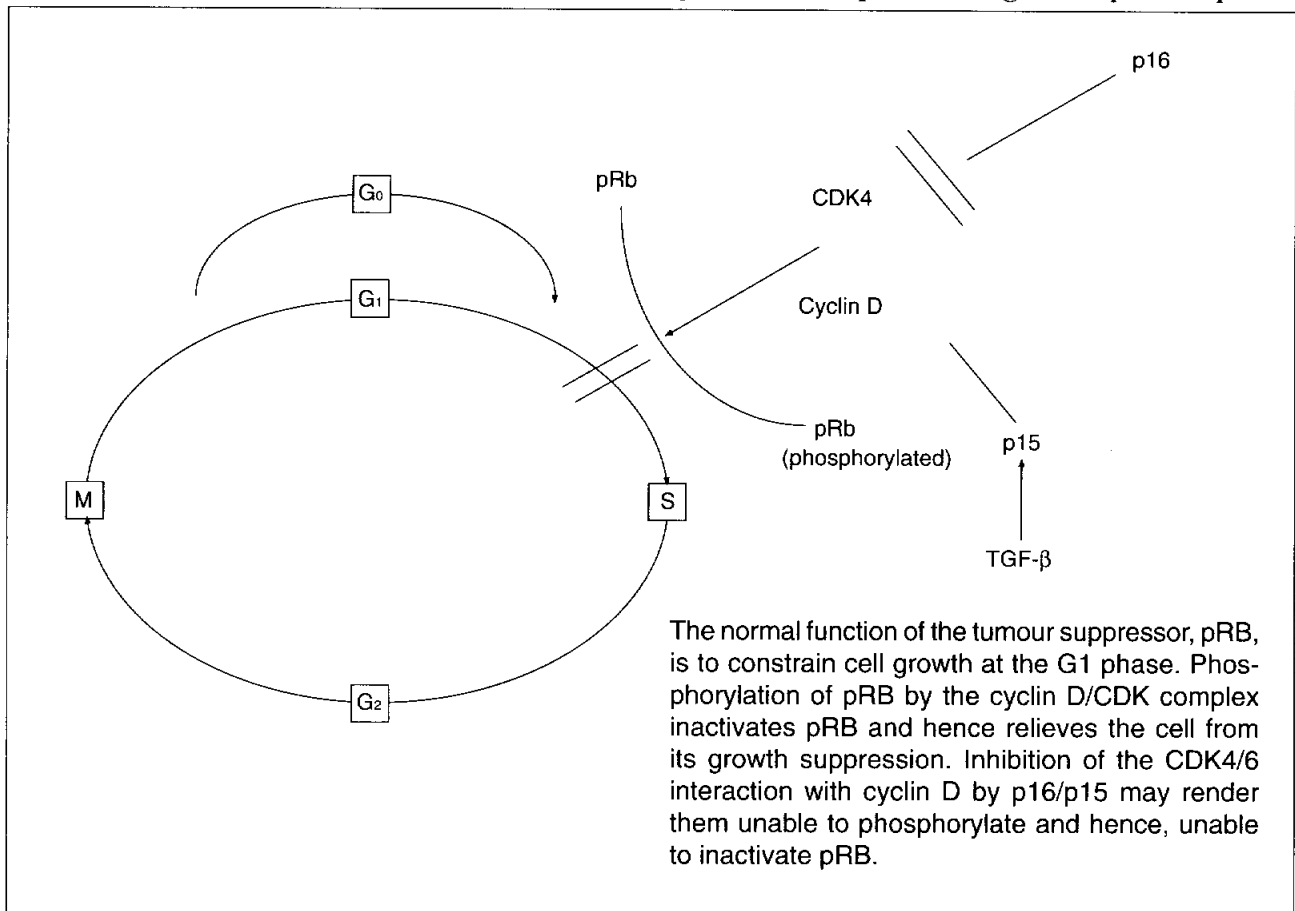
that alterations of *p16* and *p15* are involved at high incidences of MM, not by homozygous deletions or mutations, but solely by hypermethylation, which can lead to transcriptional silencing. In the study, twelve MM patients were analysed by Southern blot hybridization and polymerase chain reaction single strand conformation polymorphism analysis (PCR-SSCP); no deletions or mutations were observed. However, 75% and 67% of MMs showed hypermethylation of *p16* and *p15*, respectively. Hypermethylation of these genes was associated with blastic disease. Concomitant hypermethylation of both genes, uncommon thus far in the literature but observed in 67% of our MM cases, might be pathogenetically related to plasmacytoma development. This concomitant inactivation of both genes may be critical in eliciting the major up-stream inhibitory control of the *RB* gene in the bone marrow environment where TGF- β activity is increased. Furthermore, hypermethylation of *p16/p15* was found in both early and late stage patients and in both pre- and post-treated cases. This may suggest that they are early events in MM and their roles in tumour initiation, rather than progression, can be speculated.³⁵

Inactivation of the *p16/p15* genes, over-expression of *c-myc* and *bcl-2*, and an increase of IL-6 may be a very strong driving force for the development and progression of MM.

The genetic alterations found in nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) has a high incidence in southern China and southeast Asia but is rare in other countries. Unlike all other head and neck squamous cell carcinomas, a unique feature of this cancer is its consistent association with the Epstein-Barr virus (EBV) and the persistent detection of the EBV genome in all the nasopharyngeal carcinoma cells, regardless of their geographic, ethnic, endemic, or sporadic origin.

Epidemiology studies in the past two decades have correlated the disease with EBV infection, early age exposure to some chemical carcinogens, particularly Cantonese salted fish, and a genetically-determined susceptibility in some individuals.⁴⁰ These causal factors, in one way or another, are thought to cause multiple gene alterations, notably those involving tumour suppressor genes and proto-oncogenes. The resulting genetic damage may disturb the regulation of normal cell growth, differentiation, and apoptosis, and produce a growth advantage for a clonal population of malignant precursor cells.

Fig 2. The cell cycle is normally controlled by the *RB* gene and the up-stream regulators *p16* and *p15*

- G₀ resting phase
 G₁ first growth phase
 G₂ second growth phase
 M mitotic phase
 S synthetic phase
 pRB protein of retinoblastoma gene
 Cyclin D/CDK 4 cyclin D/cyclin-dependent kinase 4 complex
 p16 cyclin-dependent kinase inhibitor A
 p15 cyclin-dependent kinase inhibitor B
 TGF-β transforming growth factor-beta

In support of the above, early cytogenetic studies pointed to consistent chromosomal abnormalities in xenografted NPCs as well as in fresh NPC biopsies involving chromosomes 1, 3, 11, 12, and 17. These showed that multiple sites with genetic alteration may be involved in the development of NPC.⁴¹

Through molecular genetic investigations, some specific novel genetic changes have been demonstrated in NPC. Firstly, non-random and consistent loss of genetic material has been found on the short arm of chromosome 3 (67%) and chromosome 9 (61%) and the long arm of chromosome 11 (54%) at specific sites by loss of heterozygosity (LOH) analysis.⁴²⁻⁴⁶

The absence of molecular genetic material can be detected at a specific chromosomal locus in the tumour. Consistent LOH in a tumour can be used as an indication of the presence of a tumour suppressor gene and help to identify the region of the chromosome that may contain the gene. Moreover, frequent loss of heterozygosity or homozygous deletion within a specific chromosome locus in a particular tumour may suggest a tumour suppressor gene(s) in that region important for the genesis of that tumour. The presence of multiple deletion regions in several chromosomes in NPC strongly suggests the involvement of multiple tumour suppressor genes in the genesis of this cancer.

Secondly, the *RB* gene is one of the best-studied tumour suppressors, controlling cell cycle progression.⁴⁷ No remarkable gene alteration of *RB* has been detected in NPC.⁴⁸ The potential importance of *RB* alterations in NPC can thus be ruled out. On the other hand, one of the key molecular genetic alterations observed in NPC tissues is the homozygous deletion as well as hypermethylation of the *p16*/multiple tumour suppressor-1 (MTS1) gene, mapped to chromosome 9p21. This is present in the majority of NPC primary tumours (57%) examined.^{49,50} This agrees with a previous report of the frequent allele loss at chromosome 9p21 in primary NPC (61%).⁴⁵

The *p16* gene is a cell cycle negative regulator (Fig 2). It functions as a specific inhibitor of the catalytic activity of the CDK4/cyclin D complex⁵¹ and controls the traverse of a cell from the G1 to S phase of the cell cycle. Loss of *p16* gene function coupled with phosphorylation of the *RB* protein releases the E2F-DP1 transcription factors, signals entry into the S phase, and initiates uncontrolled cell proliferation. In the absence of *RB* alterations previously reported for NPC,⁴⁸ the inactivation of the *p16* gene in NPC, either through homozygous deletion or hypermethylation, are important genetic changes that affect critical cellular pathways and may thus play a crucial role in the pathogenesis and development of NPC.

The hypermethylated *p16* gene in NPC can be demethylated, using 5-aza-2'-deoxycytidine with the resulting re-expression of its mRNA in a newly established NPC cell line.⁵⁰ This indicates that an appropriate pharmaceutical drug can be used to target the methylated *p16* gene and restore its function, thus signifying a potential area for future experimental therapy.

Thirdly, the mutational inactivation of *p53* is the most frequently found molecular alteration in human cancers,⁵² but is a rare event in primary NPC (11 of 164 samples [6.7%] from southeastern China, including the provinces of Guangdong, Guangxi, Hunan, and Hong Kong, Taiwan, and Saudi Arabia).⁵³⁻⁵⁵ Over-expression of *p53* in a majority (70%-90%) of primary NPC tissues, on the other hand, has been reported.^{56,57} The *p53* over-expression does not correlate with the point mutations of the gene. However, the possibility that mutations occur in the region of the gene outside the scope of examination cannot be excluded. Moreover, the over-expression of *p53* in the majority of NPCs may also be due either to inactivation of an enzymatic pathway responsible for *p53* degradation or to stabilisation by binding with some unknown cellular or viral gene products. In any case, the role of *p53* in the development of NPC is still not clear.

In addition, the over-expression of the *bcl-2* oncogene in NPC cells has been reported.⁵⁸ This over-expression probably results from induction by the EBV latent membrane protein (LMP-1)⁵⁹; its gene product may confer an in vivo growth advantage due to prolonged survival. It has been suggested that LMP-1 helps EBV to change cell growth.²³ The enhanced cell survival induced by *bcl-2* provides an opportunity for other genetic alterations to occur, leading to tumour progression.

Lastly, the presence of telomerase activity has been examined in NPC at different clinical stages (SW Tsao, personal communication). Telomerase activation was shown to be a common event in this cancer and occurs at an early stage of tumour development. Significantly lower frequency was observed in primary NPCs with negative lymph node involvement than in those with positive lymph nodes (66.7% vs 100%, $P < 0.05$).

Conclusion

Activation of proto-oncogenes, inactivation of tumour suppressor genes, and defective DNA mismatch repair genes are believed to play essential roles in the genesis of human cancer. They act either in a specific order or as cumulative events during tumour progression, each producing a growth advantage for a clonal expanded population of cells leading to a malignant phenotype.

Areas of frequent chromosomal loss have been identified in NPCs. The genetic changes frequently found in primary NPC include specific alterations of proto-oncogenes and tumour suppressor genes, the *p16* gene in particular, as well as chromosomal losses that are thought to involve inactivation of other critical tumour suppressor genes. It has been suggested that the EBV-infected cells may be more prone to these genetic changes and result in the initiation of carcinomatous growth. However, from our recent experience, it has been observed that some of the genetic changes are already present in the atypical cells of the precancerous lesions prior to EBV infection. It is thus reasonable to propose that the development of latent EBV infection in the nasopharyngeal epithelial cells may require the presence of pre-existing specific genetic changes and that these infected cells subsequently gain a growth advantage and initiate clonal expansion towards malignancy.

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