review
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CELL CYCLE REGULATION AND HUMAN LEUKEMIAS: THE ROLE OF p16^{INK4} GENE INACTIVATION IN THE DEVELOPMENT OF HUMAN ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Recent advances in cancer biology have clearly demonstrated that the development of neoplasms as well as their progression are strictly linked to the alteration of molecular mechanisms controlling the cell division cycle. Among these mechanisms the functional inactivation of two important tumor suppressor genes, namely RB1 and p53, has been widely recognized as a pivotal step in human cancerogenesis. In addition to such well known genes, a new tumor suppressor gene, mapping on chromosome 9p21, has recently been identified and cloned. Several findings suggest that its loss of function is involved in the initiation and/or progression of an enormous number of different malignancies. This gene, named p16^{INK4}, codifies for a small protein capable of binding to, and thus of inhibiting, some specific cyclin-dependent threonine-serine kinases that represent key enzymatic activities essential for the G1-S transition in mammalian cells.

This review will summarize some aspects of the cell cycle control mechanisms, with major emphasis devoted to the role played by this recently characterized inhibitor and to the possible linkage between its inactivation and cancer formation. In particular, we will discuss these aspects in the light of the role of p16^{INK4} gene inactivation in the development of human acute lymphoblastic leukemias. Indeed this gene seems to be the first, and so far the only tumor suppressor gene consistently altered in specific acute hematological malignancies. Finally, future trends in the investigation of cell cycle control and leukemogenesis will be analyzed.

Key words: human leukemias, cell cycle, tumor suppressor genes, p16^{INK4} gene, 9p21 chromosomal alterations

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Malignant transformation is thought to be due to genetic alterations modifying the mechanisms which regulate normal cell growth and differentiation. These alterations include the somatic activation of cancer-promoting genes (cellular proto-oncogenes)[1] and the germline or somatic inactivation of tumor suppressor genes (TSGs), previously known as antioncogenes or recessive oncogenes.[2] While the identification of classical dominant oncogenes has been greatly facilitated by their ability to transform appropriate host cells,[3] the search for TSGs is remarkably complicated by the lack of strong selection procedures.

However, detailed molecular genetic studies employing restriction fragment length polymorphism or polymerase chain reaction methods,[4] along with extensive karyological analyses, have shown that non-random loss of some specific genetic material occurs in a large number of cancers and is often involved in the development or progression of the malignancy. This view is further strengthened by the observation

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of tumor suppression following monochromosome transfer in malignant cell lines.[5]

Although these studies hint at the presence of numerous TSGs, only a few recessive oncogenes have been isolated and cloned so far, such as the retinoblastoma gene (RB1 gene),[6-8] the p53 gene,[9] the neurofibromatosis gene (NF1 gene),[10] two genes mainly involved in the development of colorectal carcinoma (DCC and MCC genes),[9,11,12] a gene whose loss of function is probably responsible for Wilms' tumor (WT1),[13,14] and a gene frequently deleted in renal cell carcinoma and lung carcinoma (PTP gene).[15] Of these TSGs, RB1 and p53 are the most thoroughly characterized.

The RB1 gene was isolated and cloned in 1986[6] and was shown to include 200 kb of human chromosome 13q14.[6-8] Although its initial cloning depended on the identification of deletions involving large segments of this gene,[6-8] it soon became clear that the majority of inactive tumor-associated RB1 alleles had lost their function through subtle alterations, mostly point mutations.[8] The mutations of the RB1 gene occurring in tumor cell genomes affect the synthesis of an intact RB1 gene product (pRb1), a 105-110 kDa nuclear phosphoprotein. pRb1 is an inhibitor of cell cycle progression at G1-S by virtue of its ability to bind and thus inhibit the activity of several transcription factors, mainly E2F and possibly other HLH (helix-loop-helix) proteins (see Figure 1). The presence of E2F sites in the promoters of genes encoding thymidine kinase, *c-myc*, *c-myb*, dihydrofolate reductase, *cdc2*, and DNA polymerase α,[16-20] the products of which are strongly implicated in the

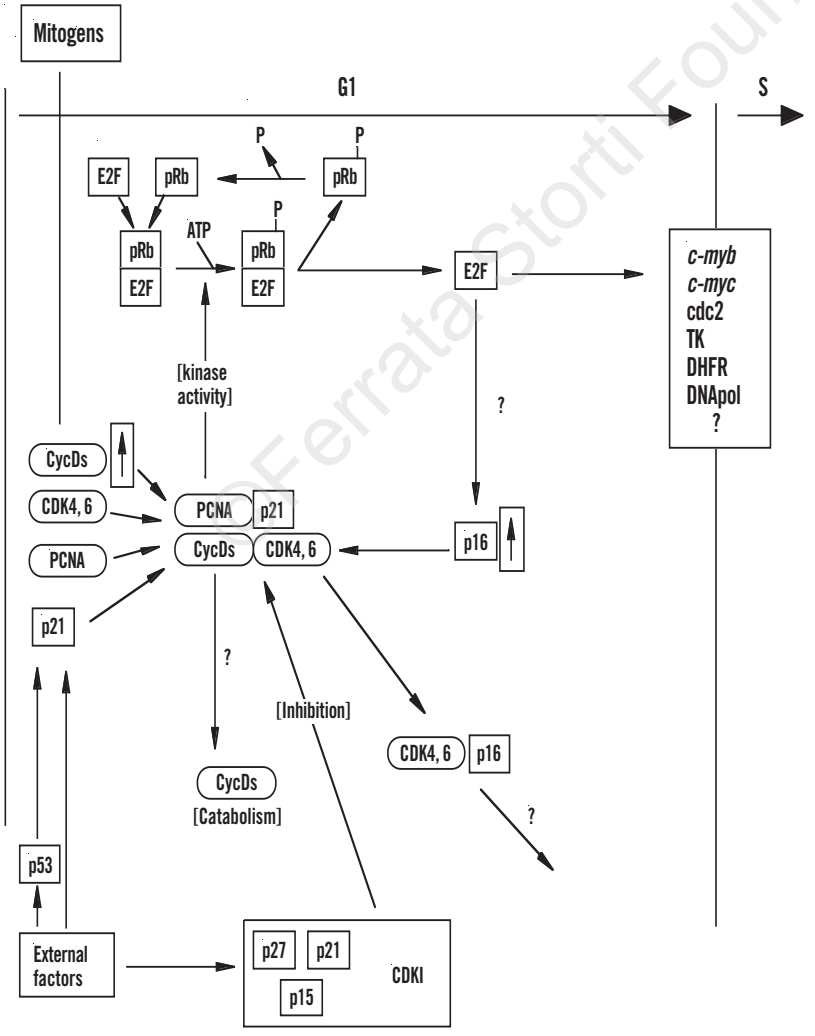

Figure 1. Regulation of mammalian G1 phase progression. D-type cyclin [CycDs] levels increase [↑] as part of the response to mitogens in cells entering the cell cycle from quiescence. $p27^{kip1}$ [p27], p21 and $p15^{INK4B}$ [p15] (CDK-inhibitors [CDKI]) levels are regulated by external factors including: ionizing radiation, TGF-β, rapamycin, Ca^{2+}, butyric acid, retinoic acid, TPA, and cAMP. The quaternary complex formed by one of the cyclin Ds, cyclin-dependent kinase [CDKs] 4 or 6, PCNA and possibly p21 is the active kinase which phosphorylates pRb1 [pRb]. Following phosphorylation of pRb1, the transcription factor E2F (or its homologues) is released and may activate several genes involved in overcoming the G1 restriction point and starting S phase (*c-myb*, *c-myc*, *cdc2*, thymidine kinase [TK], dihydrofolate reductase [DHFR], DNA polymerase a [DNApol]). Moreover, E2F may also cause upregulation of $p16^{INK4}$ [p16] gene expression. $p16^{INK4}$ protein could in turn bind to CDK 4 and 6, thus inhibiting further pRb1 phosphorylation. Finally, dissociation of the quaternary complex results in the release of cyclin Ds and in their rapid catabolism.

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induction of S phase, suggests that E2F (and its homologues) might be responsible for traversing the G1-S restriction point of the cell cycle. Starting in late G1, pRb1 is more phosphorylated than during the remainder of the cell cycle, most likely by the action of various cyclin-dependent kinases (CDKs, see the following paragraphs).

Since it is well established that only the un- or hypophosphorylated pRb1 species can bind to cellular proteins, it is highly probable that the phosphorylation of pRb1 results in a release of transcription factors from the pRb1 binding pocket and in initiation of the cell cycle.[21,22] It is also clear that this mechanism is part of a more complex control of the cell cycle since additional protein homologues to pRb1 have been isolated and E2F is only one of the proteins interacting with pRb1. The lack of a functional RB1 gene, demonstrated in several cancers, could result in constitutive activation of E2F (as well as other pRb1-interacting proteins) and in an unregulated (possibly shorter) cell cycle.

Since the TSG p53 is frequently mutated during the development of many human cancers,[23] the loss of the wild-type function of the p53 protein is thought to be a key step in carcinogenesis. Wild-type p53 suppresses both growth and transformation,[24,25] giving rise to a G1 block[26] and, in some cell types, to entry into apoptosis.[27,28] However, the molecular mechanisms involved in the inhibition of cell growth or in the activation of programmed cell death appear to be partially (or totally) independent.

Mice, which do not express functional p53, do not show any developmental defects, indicating limited participation of p53 in normal cell division and differentiation,[29] and yet the finding that these mice rapidly develop malignant cancers strongly supports a role for p53 as a TSG.[29] The exact mechanism(s) by which p53 inhibits proliferation is (are) not clear, and several functions regulating gene transcription or DNA replication have been identified that could explain the effects of the absence of p53 activity.[30]

The p53 protein acts as a transcription factor with a site-specific DNA binding domain.[31] This activity, mediated by the central portion of the protein, is due to a domain which is frequently affected by mutations in human neoplasms.[32] The C-terminus of the p53 protein contains oligomerization domains[33] that contribute to the stabilization of DNA binding activity, although this region is not required for this function.[34,35]

The p53 N-terminus is an acidic transcriptional activation domain[36] interacting with the TATA-binding protein,[37] the DNA-binding component of the general transcriptional factor TFIID. The presence of this domain results in cooperative DNA binding of p53 with the TATA-binding protein at promoters containing p53-binding sequences.[38] In addition to this specific transcriptional activation, p53 also has a more general capability of inhibiting the transcription of many different genes.[39]

Several genes activated in response to p53 have now been described and demonstrated to be regulated by promoters which contain p53-binding sites. Among these, a promising candidate for a downstream effector of p53 function is the p21 protein (see next paragraphs),[40,41] also identified as WAF1[42] or Cip-1[43] protein, a potent inhibitor of CDK activity and a component of the CDK-cyclin complexes in normal cells.[41-43] Activation of the p53 gene and the subsequent expression of p21 might cause an impairment of cell cycle progression, resulting in cell cycle arrest.[42] It must be stressed however that p21 expression has been clearly demonstrated to occur (and also to be strongly induced) in cell lines which do not express the p53 protein due to genetic inactivation of the p53 gene.[44,45] Thus, the p21 gene is regulated by several mechanisms, including (but not only) the p53 protein. In addition, further evidence suggests that p21 gene expression might be related to the activation of a cell differentiation program.[42,44,45]

This brief description of pRb1 and p53 protein functions makes it apparent that there is a strict linkage between alterations in the control of the cell cycle and tumor development. Interestingly, this represents a rational basis for development of antisense strategies in leukemia. Anti-p53 antisense oligonucleotides have been in fact employed to modulate the growth of normal and leukemic hematopoietic

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progenitors.[46]

In the next section we will describe the major molecular events involved in cell cycle division and their possible connection with cancer formation. In this context, particular emphasis will be given to the proteins that inhibit cell cycle progression.

Cell cycle division and malignant transformation

Cell cycle division

The cell cycle can be divided into four phases, namely: G1 phase (period prior to DNA synthesis), S phase (period of DNA synthesis), G2 phase (period between DNA synthesis and mitosis) and M phase (mitosis). Collectively, G1, S and G2 are called interphase, the period of the cell cycle distinct from division of the nucleus (mitosis) and cytoplasm (cytokinesis). The length of the S, G2 and M phases is remarkably similar in many different cells, while the greatest variation is seen in the length of G1. At some point late in G1, called the restriction or R point, a cell becomes committed to going through the remainder of the cycle. Thus variations in cell cycle time are mostly due to variations in the length of G1 up to the R point.

Progression through the cell cycle in eukaryotes is due to a biochemical cycle in which distinct cyclin-dependent serine-threonine kinases are sequentially activated by different cyclins. The specifically activated CDKs phosphorylate the proteinic substrates that regulate the activity and, in turn, the ordered development of cell cycle division. Thus CDK activation is the key event in cell cycle progression.

While the level of CDKs generally does not vary during the cycle, the amount of each cyclin undergoes dramatic changes. On the basis of the specific cell cycle phase in which the various cyclin levels reach their peak, it is possible to distinguish G1 phase cyclins (including cyclins Ds and E), S phase cyclins (mainly cyclins A and E) and G2 and M phases cyclins (cyclins A and Bs). The CDK partners of each cyclin are also specific, thus forming different complexes in the cell phases.[47-50] The following sequence of complexes has been postulated during cell cycle division:

phase G1: cyclins Ds (D1, D2 and D3) + CDKs (CDK2 , 4, 5, 6) →
→ phase G1 + transition G1 →S: cyclin E + CDK2 →
→ phase S + transition G2 →M: cyclin A + Cdc2 and CDK2 →
→ transition G2->M + phase M: cyclins Bs (B1 and B2) + Cdc2

According to this scheme, S phase cyclin-CDK complexes phosphorylate and activate proteins implicated in DNA replication,[48] while M phase cyclin-CDK complexes phosphorylate different proteins necessary for mitosis and cytokinesis.[49] Cyclins E and A sequentially activate CDK2 near the beginning of S phase; thus both are probably essential for initiating DNA replication.[48] In particular, cyclin A activates CDK2 shortly after cyclin E, contemporaneously with the initiation of DNA synthesis.[48]

The key component of the mitotic phase is a complex formed by the catalytic subunit Cdc2 (also defined CDK1) and its stimulatory subunits, cyclins Bs.[49] Together these form mitosis-promoting factor (MPF), an activity oscillating in the cell cycle and initiating the mitosis. Cdc2, like all other CDKs, is constitutively present; thus the accumulation and periodic destruction of cyclins determine variations in MPF activity.[49]

In mammals a tremendous number of stimuli, including mitogen agonists and antagonists, differentiaton compounds, toxic agents and extracellular matrix structural proteins act on the cells which must *decide* to proliferate, differentiate or self destruct. When activated to grow, cells react by progressing through the cycle until they reach the R point in late G1, and once past this point they will complete cell division even if external stimuli are removed.

From a biochemical point of view, as shown in the sequence reported above, there is an accumulation in the G1 phase of three D-type cyclins (D1, D2 and D3) which assemble into holoenzymes with either CDK4 or CDK6 (Figure 1).[47,50] Interaction between cyclins Ds and these CDKs is not sufficient to activate the

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kinase subunit of the holoenzyme, since functioning CDKs must be phosphorylated at a single threonyl residue and dephosphorylated at a specific tyrosine residue. This points to novel protein kinases and/or phosphatases as key regulators of G1-CDKs complexes.

Additional proteins are normal constituents of complexes formed between D-type cyclins and CDK4 (or CDK6); these include the proliferating cell nuclear antigen (PCNA) and p21 (Figure 1). PCNA, the processing factor required by eukaryotic DNA polymerases δ and ϵ, is involved in the control of the rate of chromosome replication as well as in the repair of damaged DNA.[51,52]

The precise amount of PCNA-containing multisubunit complexes with distinct functions in each cycle phase is not known, but their presence strongly suggests a strict linkage between the cell cycle and the replication and repair of DNA. The main features of protein p21 are described elsewhere in the review, but at this point it should be noted, in the context of cell cycle regulation, that the nature and the precise stochiometry of all the complexes involving p21 are not clear. Indeed, it is totally possible that there might be different multisubunit p21-containing holoenzymes with distinct catalytic properties.

Only a few substrates of active CDK4 and CDK6 have been identified, such as the tumor suppressor gene, pRb1 (and its homologues). As stated in the introduction, at the beginning of the G1 phase this TSG protein is in a dephosphorylated form and is bound to various transcription factors, including E2F. When pRb1 is phosphorylated by active CDK4 or CDK6, the TSG complexes are dissociated and E2F (or its functional analogues) is released and is free to activate the genes involved in starting DNA duplication and in the progression from G1 to S phase (Figure 1).

Cell cycle and cyclin-dependent kinase inhibition: the p16^{INK4} connection

The enzymatic activity of a CDK is regulated at three different levels: cyclin binding and activation, subunit phosphorylation, and association with and inhibition by a group of heterologous small regulatory proteins. In the previous paragraph we described binding with specific cyclins and the CDK activation by phosphorylation. In this section we will describe the recently cloned small cell-cycle regulatory proteins that have been identified by virtue of their ability to interact physically with cyclin or CDK proteins.

A 21 kDa protein, p21, was initially identified in anti-cyclin D1 immunoprecipitates of human diploid fibroblasts as a component of quaternary cyclin Ds complexes that also contained CDKs and PCNA. Subsequently it was discovered to be a potent inhibitor of all known cyclin-CDK holoenzymes[40-43] and a target of p53 transcriptional activation (see the ***Introduction*** for additional details).

p21 is a negative regulator of all CDK multisubunit complexes, including those kinases involved in the progression of the cycle through the S, G2 and M phases.[40-43,53] It is a normal constituent of these holoenzymes in their active form,[40] but when the level of p21 increases the stochiometry of the complexes changes and kinase activity is inhibited.[41] p21 is also able to bind PCNA directly, thereby modifying its activity in DNA replication but not in DNA repair.[54]

A protein inhibitor strictly related to p21, namely p27^{Kip1}, was also found to bind to and thus inhibit various cyclin-CDK enzymatic activities. This protein was postulated to be the potential mediator of G1 cell-cycle arrest caused by TGF-β treatment or by cell-cell contact inhibition.[55-57] So far, no important direct connections have been demonstrated between the absence or the alteration of p21 or p27^{Kip1} and malignant transformation, although it is highly possible that in the future such a linkage could be shown.

Another key regulator protein of CDK activity, in addition to the above-cited p21 and p27^{Kip1}, is a peptide named p16^{INK4}. Indirect evidence demonstrated that this protein is able to bind CDK4 or CDK6 and at the same time dissociate complexes formed by cyclin D-CDK-p21-PCNA (Figure 1). Two main functional differences appear to exist between p21 and p16^{INK4}, namely: i) p21 is expressed mainly as a

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consequence of external stimuli (either dependent on or independent of p53 gene activation) which inhibit cell growth[41,42] and/or might cause differentiation,[44,45] while p16^{INK4} seems to belong to an intrinsic regulatory loop mainly related to pRb1 phosphorylation status[58] (Figure 1); ii) p21 forms complexes containing CDK and other proteins, while p16^{INK4} binds exclusively to CDK4 and CDK6, thus destroying the active quaternary complexes.

Considerable attention has recently been paid to p16^{INK4} mainly in relation to the close link between p16^{INK4} alterations and cancer development. This strong relationship involves the localization of the p16^{INK4} gene on the short arm of chromosome 9, more precisely in the region 9p21.[59] This chromosomal area is of great interest since a tremendous number of studies have clearly demonstrated that a putative TSG possibly implied in the development of a large variety of different cancers is localized in this region. Successively, several studies indicated the p16^{INK4} gene as the probable TSG.[60-68]

Two important features are specific for p16^{INK4} with respect to p53 as a TSG: i) the p16^{INK4} gene is usually inactivated by homozygous deletions[63-68] and not, as occurs for the p53 gene, by point mutations;[23] ii) the structure of the p16^{INK4} protein is formed by several (four) repeats of ankyrin motifs, which is a characteristic that often occurs in a large number of peptides involved in protein-protein interaction.[59] Conversely, p53 acts essentially as a transcription factor.[31]

It is important to underline that the 9p21 deletions might also involve a gene strictly linked to p16^{INK4}, referred to as p15^{INK4B} (also known as p14^{INK4B}).[61,69,70] Such a gene codifies for a protein which shows a high degree of structural similarity with p16^{INK4} as well as a similar mechanism of action. However, p15^{INK4B} gene expression seems to be up-regulated by external cellular stimuli[69] while p16^{INK4} appears to be an intrinsic brake to cell proliferation.

In the next section we will summarize the main data reported in the literature on TSGs and human leukemias, especially those on the role of alterations in p16^{INK4} gene in these types of neoplasms.

Cyclin-dependent kinase inhibitors and human leukemias

Tumor suppressor genes in human leukemias

Remarkable progress has been made recently in the identification of specific growth regulatory genes (dominant oncogenes and TSGs) involved in the development and progression of human leukemias, and of the mechanisms by which their function is altered. The latter comprise changes in gene location following chromosome rearrangements, major additions or deletions of genetic material, point mutations and other mechanisms. Generally, chromosome translocation represents, at least in human leukemias, a major mechanism for *activation* of cellular proto-oncogenes. For example, in nearly every typical case of chronic myelogenous leukemia, the neoplastic cells show only a t(9;22) translocation that produces the Philadelphia (Ph) chromosome. In this rearragment, the *c-ABL* proto-oncogene is translocated from its normal site on chromosome 9, band q34, to a very restricted region on chromosome 22, the breakpoint cluster region (BCR).[71]

Additional examples include human lymphoid tumors. Among B-cell lymphomas (in particular Burkitt's tumor), a t(8;14) characterizes 75% of these neoplasms. This translocation brings the immunoglobulin heavy-chain locus at chromosome band 14q32 into juxtaposition with the c-myc proto-oncogene, normally located on chromosome band 8q24. These studies have also been extended to other B-cell neoplasms which show various specific genomic alterations, namely t(14;18) (involving bcl-2 gene), t(1;19) and t(14;18), and to acute promyelocytic leukemia which presents t(15;17).[72-74]

The only potentially identified TSG in human leukemias is the p53 gene,[72] that appears to be mutated in about one third of acute lymphoblastic leukemias (ALL) of T-cell origin in the relapse phase of the disease while almost no p53 mutations are found at presentation of T-ALLs. This suggests that the genetic alteration might be related to the clinical progression of this neoplastic pathology. Interestingly, RB1 does not appear to be remarkably modified in human leukemias; however, some additional

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non-random deletions in myeloid and lymphoid tumors have been identified, including chromosomes 6 (6q–), 7 (7q–) and 9 (9p–), suggesting the involvement of several TSGs in these neoplasms.[72] The next paragraph will describe available data on the frequency and meaning of 9p21 aberrations.

Chromosome 9p21 genetic deletions and acute lymphoblastic leukemias

Acute lymphoblastic leukemia is a heterogeneous group of neoplasms that are caused by a clonal expansion of the precursors of B or T lymphocytes. Although progress in ALL therapy has led to induction of remission in nearly all patients, about 30 to 50% of cases still have relapses. This last event, namely relapse, is particularly dreaded since intractable disease develops in most patients in whom it occurs. Various clinical and laboratory features present at the time of diagnosis allow one to predict the likelihood that a patient will remain in remission. These prognostic factors include white-cell count, age and sex, and the extent of bulky disease in sites such as lymph nodes, spleen and mediastinum. In addition, it is now recognized that chromosomal pattern may be an independent prognostic feature.

Among patients with ALL, deletions or unbalanced translocations of the short arm of chromosme 9 have been reported with frequencies of 7 to 13%75-77. The smallest segment that is lost includes band 9p21.

Since these discoveries, detailed molecular biology analyses of chromosome 9p have been carried out by both classical restriction fragment length polymorphism studies and linkage analyses employing polymerase chain reaction amplification of selected regions. The main probes of the region included cDNA of the a-interferon (IFNA) gene cluster, the β-interferon (IFNB1) locus, the glycoprotein 4b-galactosyltransferase (GGTB2) gene, the tyrosinase-related protein (TYRP) gene, and the arginin-succinate synthase pseudogene 3 (ASSP3)[59] 4and references therein).

Additional markers of the 9p chromosome were D9S33, D9S126, D9S3 and D9S19. With the aid of all these probes a very detailed genetic and physical map of the region surrounding the interferon genes on 9p was created, with the following suggested order: D9S33, IFNB1, IFNA, D9S126, D9S3, D9S19, GGTB2 and ASSP3[59] (and references therein). The most important information came from studies on the absence of the enzyme 5'-deoxy-5'-methylthioadenosine phosphorylase (MTAPase) whose codifying gene was shown to map strictly linked to the putative 9p21 TSG.[59]

Using all the probes mentioned and taking into consideration the order of the genes on the 9p chromosome, several groups investigated the genetic alterations of this areaat the molecular level, and finally in April 1994 two independent research groups identified the putative 9p21 TSG as the previously cloned p16INK4 gene.[60,61]

It is important to emphasize that this gene was characterized at the end of 1993 as a specific inhibitor of CDK4 and CDK6, totally ignoring its role as a TGS.[59] Thus, the convergence of two important independent research areas, namely studies on the cell cycle and investigations on tumor biology, resulted in the identification of this new key TSG.

5'-deoxy-5'-methylthioadenosine phosphorylase deficiency

As stated before, an important contribution to the identification of the $p16^{INK4}$ gene as the TSG occurring at chromosome 9p21 derived from studies on MTAPase deficiency in human tumors. MTAPase (5'-deoxy-5'-methylthioadenosine: orthophosphate methylthioribosyltransferase, EC 2.4.2.28) catalyzes the phosphorolytic cleavage of 5'-deoxy-5'-methylthioadenosine (MTA), a sulfur adenosyl nucleoside formed from S-adenosylmethionine through several independent pathways.[78-80] The reaction products, adenine and 5-methylthioribose l-phosphate, are then recycled to AMP and methionine, respectively.[78-80] Therefore the enzyme presumably plays a key role in a purine salvage pathway and in the recycling of methylthio groups.[78-80]

Due to its central role in mammalian metabolism, this enzymatic activity has been found in all normal tissues and cell lines of non-malignant origin investigated so far. Conversely, a

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large number of studies since 1981 have shown that a high percentage of human cell lines established from different tumors completely lack MTAPase activity.[78-80] Moreover, patients with MTAPase-negative tumors have normal enzymatic activity in nonmalignant cells of the same lineage, thus indicating that these subjects were not genetically phosphorylase-deficient and that the deficiency was acquired during cancer development.[81] It should be underlined, in the context of this review, that this last study was carried out on human leukemias and that the MTAPase deficiency was demonstrated in 10% of the acute leukemias analyzed and, in particular, in 38% of T-cell ALL cases.[81]

By means of mouse-human somatic cell hybridization studies, the MTAPase gene was preliminarily mapped on the 9pter-9ql2 region[82] and more recently on 9p21[60] at about 80 kb from the p16^{INK4} gene. It is now clear that the absence of MTAPase activity in such a large number of tumors is due to two concomitant events: i) the strict linkage between the phosphorylase and the TSG gene on the 9p21 chromosome, and ii) the mechanism of p16^{INK4} gene inactivation that is for the most part secondary to the deletion of genetic materials.

The absence of MTAPase activity in human tumors and in particular in leukemias should not be considered as merely an epiphenomenon of the process of p16^{INK4} gene inactivation, but as an important observation in view of its therapeutical consequences. Indeed, since the cleavage of MTA is the only endogenous source of adenine for the enzyme adenine phosphoribosyltransferase, administration of the thioether represents an ideal route for supplying the purine ring in normal MTAPase-containing cells. This could be of pivotal importance when a purine synthesis inhibitor is employed in a therapeutic cancer protocol.

Some of these schemes have been previously reported[83,84] and are mainly based on the role of the enzyme in the recycling of the adenine and methionine moieties from MTA. In particular, it has been proposed that the contemporaneous administration of a purine synthesis inhibitor (like azaserine, methotrexate or the more specific 5,10-dideazatetrahydrofolate) and MTA could result in a selective killing of enzyme-negative cells.[83,84] On the contrary, the normal MTAPase-containing cells should be protected from the toxic effects of the drug by the intracellular release of the adenine moiety due to the cleavage of MTA. This therapeutic approach could be especially useful in the context of bone marrow purging before autologous transplantation. Further investigations are necessary to develop and test the above mentioned strategy as well as other new selective therapeutic protocols based on MTAPase deficiency.

p16^{INK4} gene deletions in human leukemias

After the p16^{INK4} gene was determined to be the most probable candidate for the TSG mapping on the 9p21 chromosome, several research groups[65-67,84] started to investigate the incidence of the deletion of this gene in human leukemias. Tables 1 and 2 summarize some of the data reported in the literature including the analysis of deletions in 37 established cell lines[65] (Table 1) and in a large number of patients (>200 cases) with different hematologic neoplasms[65-67] (Table 2). In addition, a detailed analysis involving only childhood acute leukemias was carried out in the authors' laboratories and is reported in Table 3.[85,86]

Several conclusions can be reached by analyzing the data reported in these tables. First of all, the p16^{INK4} gene is consistently deleted in ALL, while the incidence of its deletion in other hematological neoplasms is very low (or not found at all). Secondly, among ALL types, the T-cell-derived neoplasms show a high percentage of deletions. This can be seen in both adult and

Table 1. p16^{ink4} gene deletion in human leukemic cell lines.*

Cell line type	homozygous p16^{INK4} gene deletions	Percentage (%)
Myelocytic/monocytic	1/11	9
Erythroid	3/4	75
Megakaryocytic	2/4	50
B-lymphocytic	3/11	27
T-lymphocytic	5/7	71

**The data reported are taken from Ref. 65.*

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Table 2. p16[INK4] gene deletion in human leukemias.

Disease	homozygous p16[INK4] gene deletions	Percentage (%)
AML*	0/45	0
AML**	0/11	0
ALL*	4/14	28
T-ALL**	3/12	25
T-ALL***	20/24	83
B-ALL**	6/39	15
B-ALL***	2/31	6
Burkitt's ALL	0/12	0
CML-BC*	0/13	0
CLL**	0/20	0
MDS**	0/28	0
Myeloma **	0/18	0

*The data reported are taken from: *, Ref. 65; ** Ref. 67; *** Ref. 66. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML-BC, chronic myeloid leukemia in blastic crisis; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndromes.*

childhood ALL. Finally, at least in pediatric ALLs, the cancers derived from T and B precursors seem to involve a preponderance of homozygous deletions.

On the other hand, very few p16[INK4] gene point mutations have been demonstrated, at least in hematological neoplasms, thus suggesting an intrinsic fragility in the 9p21 region.[65-67] It should also be pointed out that a large number of deletions have been detected in ALL at presentation. This could mean that this genetic abnormality is involved in the early phases of ALL development. Moreover, the apparent lineage specificity implies that the p16[INK4] protein may be an important factor in the control of cell phenotype. This aspect will require very thorough investigation in the future.

Table 3. p16[ink4] gene deletion in childhood human leukemias.

Disease	homozygous p16[INK4] gene deletions	Percentage (%)
AML	0/4	0
Common-ALL	6/13	46
Early-T-ALL	3/3	100
Pre-B-ALL	2/2	100

**The data reported are taken from Ref. 65.*

As stated before, p53 is the only other TSG consistently identified in human ALL; however to our knowledge its loss of function has been demonstrated at presentation in only one case.[87] Thus, at the moment, the p16[INK4] gene remains the only TSG significantly inactivated in human ALL at presentation.

Future scenarios

Several possible avenues of research could be hypothesized on the basis of the findings reported in the previous sections.

First of all, a pivotal aim of research will be to understand the true role of the p16[INK4] protein in the control of cell proliferation. Indeed it is clear that none of the small CDK-inhibitors (p16[INK4], p15[INK4B], p21 or p27[*Kip1*]) can be thought of as the sole controllor of the rate of cellular growth.

Conversely, it is possible that p16[INK4] acts, probably together with additional proteins, as an intrinsic brake on cell cycle division by regulating the activity of CDK4 and CDK6 at specific cycle phases. Additional CDK-inhibitors, like p21, p27[*Kip1*], p15[INK4B] and others might represent intracellular effectors of external stimuli that could also induce differentiation. This hypothesis however needs experimental confirmation, since no data on the variation of p16[INK4] gene expression in the normal cell cycle are available. Concerning the role of p16[INK4] gene inactivation in cancerogenesis, several aspects merit intensive future studies. It is highly probable that the loss of function of this new TSG occurs as an early step in the malignant transformation process and possibly causes faster cell cycle division. This could in turn stimulate further genetic alterations and progression towards (a) more malignant genotype(s). In this context, the finding that p16[INK4] gene inactivation occurs with an extremely high incidence in specific tumor types (such as some subsets of ALL) raises the question of whether this protein is particularly important for the growth and differentia-

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tion of specific tissues like the immunological system. It is intriguing that a certain degree of homology has been demonstrated between the $p16^{INK4}$ protein and genes involved in cell differentiation (Notch gene).[70] Thus $p16^{INK4}$ could play additional, as yet uninvestigated roles in specific cell types.

An additional consideration regards the mechanism of $p16^{INK4}$ gene inactivation which appears to be mostly due to genomic deletions. An intrinsic 9p21 fragility, which could be the cause of the large number of tumors showing this aberration, has been reported. However, as discussed before, of the hematological neoplasms only ALL present 9p21 deletions; thus the intrinsic instability is cell lineage specific. Alternatively, it is possible that loss of $p16^{INK4}$ gene function results in specific cell differentiation towards a particular cell lineage. It will also be interesting to characterize the genomic area involved in the deletion process.

Some important clinical considerations must also be made. $p16^{INK4}$ represents the first TSG characterized in human acute lymphoblastic leukemias at presentation. Although this aberration appears to be involved in the early phase of hematological neoplasm formation, future studies are needed to substantiate this hypothesis. In addition, it will be essential to clarify the prognostic value of this new genetic alteration.

Another aspect to consider is related to the potential role of $p16^{INK4}$ gene inactivation in ALL therapy. One area of study will be to identify molecules (natural compounds or small peptides) that are able to mimick $p16^{INK4}$ and thus ameliorate the patient's clinical condition. A second area of studies, in our opinion, is related to the possibility of utilizing the deficiency of genes linked to the $p16^{INK4}$ gene (namely the MTAPase gene) to develop new therapeutical strategies.

Finally, given the rapid rate of progress, it is easy to predict that this field will be ready for a new review in a very short time.

References

1. Bishop JM. The molecular genetics of cancer. Science 1987; 235:305-11.
2. Weinberg RA. Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. Cancer Res. 1989; 49:3713-21.
3. Reddy EP, Skalka AM, Curran T. The Oncogene Handbook. Amsterdam: Elsevier Science Publ., 1988.
4. Ponder B. Gene losses in human tumours. Science 1988; 335:400-2.
5. Sager R. Tumor suppressor genes: the puzzle and the promise. Science 1989; 246:1406-12.
6. Friend SH, Bernards R, Rogeij S, et al. A human DNA segment with properties of the gene that predisposes to retinoblastoma. Nature 1986; 323:643-6.
7. Lee WH, Bookstein R, Hong F, Young L-J, Shew J-Y, Lee EY-HP. Human retinoblastoma susceptibility gene: cloning identification and sequence. Science 1987; 235:1394-9.
8. Fung Y-KT, Murphree AL, T'Ang A, Qian J, Hinrichs SH, Benedict WF. Structural evidence for the authenticity of the human retinoblastoma gene. Science 1987; 236:1657-61.
9. Baker SJ, Fearon ER, Nigro JM, et al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 1989; 244:217-21.
10. Viskohil D, Buchberg A, Xu G, et al. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 1990; 62:187-92.
11. Fearon ER, Cho KR, Nigro JM, et al. Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 1990; 247:49-56.
12. Kinzler KW, Nilbert MC, Su LK, et al. Identification of FAP locus gene from chromosome 5q21. Science 1991; 253:661-5.
13. Rose EA, Glaser T, Jones C, et al. Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms' tumor gene. Cell 1990; 60:495-508.
14. Call KM, Glaser T, Ito CY, et al. Isolation and characterization of a zinc-finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 1990; 60:509-20.
15. LaForgia S, Morse B, Levy J, et al. Receptor protein-tyrosine phosphatase γ is a candidate tumor suppressor gene at human chromosome region 3p21. Proc Natl Acad Sci USA 1991; 88:5036-40.
16 Johnson PF, McKnight SL. Eukaryotic transcriptional regulatory proteins. Annu Rev Biochem 1989;58:799-839.
17. Hiebert SW, Lipp M, Nevins JR. E1A-dependent trans-activation of the human MYC promoter is mediated by the E2F factor. Proc Natl Acad Sci USA 1989; 86:3594-8.
18. Blake MC, Azizhan JC. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene *in vitro* and *in vivo*. Mol Cell Biol 1989; 9:4994-5002.
19. Dalton S. Cell cycle regulation of the human cdc2 gene. EMBO J 1992; 11:1797-804.
20. Pearson BE, Nasheuer HP, Wang TS-F. Human DNA polymerase alpha gene: sequences controlling expression in cycling and serum-stimulated cells. Mol Cell Biol 1991; 11:2081-95.
21. Nevins JR. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science 1992; 258:424-9.
22. Sherr CJ. Mammalian G1 cyclins. Cell 1993; 73:1059-65.
23. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1993; 253:49-53.
24. Baker SJ, Markowits S, Fearon ER, Wilson JKV, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990; 249:912-5.
25. Finlay CA, Hinds P, Levine AJ. The p53 protooncogene can act as a suppressor of transformation. Cell 1989; 57:1083-93.
26. Lin D, Shields MT, Ullrich SJ, Appella E, Mercer WE. Growth arrest induced by wild-type p53 blocks cells prior or near the restriction point in late G1 phase. Proc Natl Acad Sci USA 1992; 89:9210-4.

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27. Bergamaschi G, Rosti V, Danova M, Cazzola M. Apoptosis: biological and clinical aspects. Haematologica 1994; 79:86-93.
28. Ryan JJ, Danish R, Gottlieb CA, Clarke MF. Cell cycle analysis of p53-induced death in murine erythroleukemia cells. Mol Cell Biol 1993; 13:711-9.
29. Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992; 356:215-21.
30. Pientenpol JA, Vogelstein B. No room at the p53 inn. Nature 1993; 365:17-8.
31. Kern SE, Kinzler KW, Bruskin, et al. Identification of p53 as a sequence-specific DNA binding protein. Science 1991; 252:1708-11.
32. Bargonetti J, Manfredi JJ, Chen X, Marshak DR, Prives C. A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Genes Dev 1993; 7:2565-74.
33. Pavietich NP, Chambers KA, Pabo CO. The DNA-binding domain of p53 contains the four conserved regions and the major hot spots. Genes Dev. 1993; 7:2556-64.
34. Hupp TR, Meek DW, Midgley CA, Lane DP. Regulation of the specific DNA binding function of p53. Cell 1992; 71:875-86.
35. Tarunina M, Jenkins JR. Human p53 binds DNA as a protein homodimer but monomeric variants retain full transcriptional transactivating activity. Oncogene 1993; 8:3165-73.
36. Fields S, Jang SK. Presence of a potent transcription activating sequence in the p53 protein. Science 1990; 249:1046-9.
37. Liu X, Miller CW, Koffler PH, BerkAJ. The p53 activating domain binds the TATA box-binding polypeptide in holo-TFIID, and a neighboring p53 domain inhibits transcription. Mol Cell Biol 1993; 3291-300.
38. Chen X, Farmer G, Zhu H, Prywes R, Prives, C. Cooperative DNA binding p53 with TFIID (TBP): a possible mechanism for transcriptional activation. Genes Dev 1993; 7:1837-49.
39. Ginsberg D, Mechta F, Yaniv M, Oren M. Wild-type p53 can down-modulate the activity of various promoter. Proc Natl Acad Sci USA 1991; 88:9979-83.
40. Xiong Y, Zhang H, Beach D. D-type cyclins associated with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 1992; 71:505-14.
41. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993; 366:701-4.
42. El-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potent mediator of p53 tumor suppression. Cell 1993; 75:815-25.
43. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge S. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993; 75:805-16.
44. Steiman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA, El-Houssein ME. Induction of p21 (WAF-1/CIP1) during differentiation. Oncogene 1994; 9:3389-96.
45. Jiang H, Lin J, Su Z-z, Collart FR, Huberman E, Fisher PB. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene 1994; 9:3397-406.
46. Martinelli G, Ferrari S. Meeting report: antisense oligonucleotides. Haematologica 1994; 79:184-8.
47. Sherr CJ. G1 phase progression: cycling on cue. Cell 1994; 79:551-5.
48. Heichman KA, Roberts JM. Rules to replicate by. Cell 1994; 79:557-62.
49. King RW, Jackson PK, Kirschner MW. Mitosis in transition. Cell 1994; 79:563-71.
50. Hunter T, Pines J. Cyclins and cancer II: cyclin D and CDK inhibitors. Come of age. Cell 1994; 79:573-82.
51. Prelich G, Tan C-K, Kostura M, et al. Functional identity of proliferating cell nuclear antigen and a DNA polymerase auxilliary protein. Nature 1987; 326:517-20.
52. Morrison A, Araki H, Clark AB, Hamatake RK, Sugino A. A third essential DNA polymerase in *S. cerevisiae*. Cell 1990; 62:1143-51.
53. Gu Y, Turk CW, Morgan DO. Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. Nature 1993; 366:707-10.
54. Waga S, Hannon G, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 1994; 369:574-8.
55. Firpo EJ, Koff A, Solomon MJ, Roberts JM. Inactivation of Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. Mol Cell Biol 1994; 14:4889-901.
56. Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. EMBO J 1994; 11:2909-17.
57. Polyak K, Lee M-H, Erdjument-Bromage H, et al. Cloning of p27kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 1994; 78:59-66.
58. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. Nature 1993; 266:122-6.
59. Della Ragione F, Oliva A, Palumbo R, Russo GL, Zappia V. Enzyme deficiency and tumor suppressor genes: absence of 5'-deoxy-5'-methylthioadenosine phosphorylase in human tumors. Adv Exp Med Biol 1993; 348:31-43.
60. Nobori T, Miura K, Wu A, Luis K, Takabashi K, Carson DA. Deletion of the cyclin-dependent kinase 4 inhibitor gene in multiple human cancers. Nature 1994; 368:753-6.
61. Kamb A, Gruis NA, Weaver-Feldhaus J, et al. A cell cycle regulatory potentially involved in genesis of many tumor types. Science 1994; 264:436-40.
62. Kamb A, Shattuck-Eidens D, Eeles R, et al. Analysis of the p16 gene [CDKN2] as a candidate for the chromosome 9p melanoma susceptibility locus. Nature Genet 1994; 8:22-6.
63. Caldas C, Hahn SA, da Costa LT, et al. Frequent somaticmutations and homozygous deletions of the p16 [MTS1] gene in pancreatic adenocarcinoma. Nature Genet 1994; 8:27-32.
64. Cairns P, Man L, Merlo A, et al. Rates of p16 (MTS1) mutations in primary tumors with 9p loss. Science 1994; 265:415-6.
65. Ogawa S, Hirano N, Sato N, et al. Homozygous loss of cyclin-dependent kinase 4-inhibitor (p16) gene in human leukemias. Blood 1994; 84:2431-5.
66. Hebert J, Cayuela JM, Berkeley J, Sigaux F. Candidate tumor-suppressor genes MTS1 ($p16^{INK4A}$) and MTS2 ($p15^{INK4B}$) display frequent homozygous deletions in primary cells from T- but not from B-cell lineage acute lymphoblastic leukemias. Blood 1994; 8:4036-44.
67. Quesnel B, Preudhomme C, Philippe N, et al. p16 gene homozygous deletions in acute lymphoblastic leukemia. Blood 1995; 85:657-63.
68 Della Ragione F, Russo GL, Oliva A, et al. 5'-Deoxy-5'-methylthioadenosine phosphorylase and $p16^{INK4}$ deficiency in multiple tumor cell lines. Oncogene 1995; 10:827-33.
69. Hannon GJ, Beach D. $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. Nature 1994; 371:257-60.
70. Guan K-L, Jenkins CW, Ki Y, et al. Growth suppression by p18, a $p16^{INK4}$/MTS1 and $p14^{INK4B}$/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev 1994; 8:2939-52.
71. Witte ON, Kelliher M, Muller AJ, et al. Role of bcr-abl oncogene in the pathogenesis of Philadelphia chromosome positive leukemias. In: Brugge J, Curran T, Harlow E, McCormick F, eds. Origins of human cancers. Cold Spring Harbor:Cold Spring Harbor Laboratory Press, 1992:521-6.
72. Nowell PC. Origins of human leukemia: an overview. In Brugge J, Curran T, Harlow E, McCormick F, eds. Origins of

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human cancers. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1992:513-20.
73. Croce CM. Molecular biology of leukemias and lymphomas. In: Brugge J, Curran T, Harlow E, McCormick F, eds. Origins of human cancers. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1992:527-42.
74. Borow J, Goddard AD, Sheer D, Solomon E. Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science 1990; 249:1577-80.
75. Kowalczyk J, Sandberg AA. A possible subgroup of ALL with 9p-. Cancer Genet Cytogenet 1981; 9:383-5.
76. Chilcote RB, Brown E, Rowley JD. Lymphoblastic leukemia with lymphomatous features associated with abnormalities of the short arm of chromosome 9. N Engl J Med 1985; 313:286-91.
77. Diaz MO, Rubin CM, Harden A, et al. Deletions of interferon genes in acute lymphoblastic leukemia. N Engl J Med 1985; 322:77-82.
78. Della Ragione F, Cartenì-Farina M, Gragnaniello V, Schettino MI, Zappia V. Purification and characterization of 5'-deoxy-5'-methylthioadenosine phosphorylase from human placenta. J Biol Chem 1986; 261:12324-9.
79. Della Ragione F, Oliva A, Gragnaniello V, Russo GL, Palumbo R, Zappia V. Physicochemical and immunological studies on mammalian 5'-deoxy-5'-methylthioadenosine phosphorylase. J Biol Chem 1990; 265:6241-6.
80. Della Ragione F, Cartenì-Farina M, Zappia V. 5'-deoxy-5'-methylthioadenosine: novel metabolic and physiological aspects. In: Bachrach U, Heimer YM eds. The physiology of polyamines. Boca Raton, Florida: CRC Press, Inc., 1989:231-54.
81. Traweek ST, Riscoe MK, Ferro AJ, Braziel RM, Magenis RE, Fichten JH. Methylthioadenosine phosphorylase deficiency in acute leukemia: pathologic, cytogenetic, and clinical features. Blood 1988; 71:1568-73 .
82. Carrera CJ, Eddy RL, Shows TB, Carson DA. Assignment of the gene for methylthioadenosine phosphorylase to human chromosome 9 by mouse-human somatic cell hybridization. Proc Natl Acad Sci USA 1984; 81:2665-78.
83. Kamatani N, Nelson-Rees WA, Carson DA. Selective killing of human malignant cell lines deficient in methylthioadenosine phosphorylase, a purine metabolizing enzyme. Proc Natl Acad Sci USA 1981; 78:1219-23.
84. Nobori T, Szinai I, Amox D, et al. Methylthioadenosine phosphorylase deficiency in human non-small cell lung cancers. Cancer Res 1992; 53:1098-101.
85. Iolascon A, Perrotta S, Mastropietro S, et al. Homozygous deletions of p16INK4 gene in childhood acute leukemias. Blood 1994; 84:(suppl. 1):298a.
86. Iolascon A, Faienza MF, Coppola B, Della Ragione F, Santoro N, Schettini F. High frequency of homozygous deletions of CDK4I gene in childhood acute lymphoblastic leukaemia. Br J Haematol 1995; (in press).
87. Diccianni MB, Yu S, Hsiao M, Mucherjee S, Shao LE, Yu AL. Clinical significance of p53 in relapsed T-cell acute lymphoblastic leukemia. Blood 1994; 84:3105-12.

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