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Oncogenes and Tumor Suppressor Genes: New Biochemical Tests

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ABSTRACT: Oncogenes and tumor suppressor genes are genes involved in cell proliferation and differentiation. They play a crucial role in the initiation and progression of cancer. Some of these genes are found to be altered in human cancers, i.e., mutated, amplified, deleted, translocated, or abnormally regulated. Recently, the protein products of the genes have been purified, and antibodies against them have been developed. Studies of oncogenes and tumor suppressor genes at the DNA, mRNA, or protein level may reveal new ways for diagnosis, monitoring, prognosis, and treatment of cancer. In this article, the area of oncogenes and tumor suppressor genes is reviewed, with emphasis on clinical applications and biochemical testing. Although most of the currently known genetic markers are not sufficiently specific or sensitive, it is anticipated that the discovery of newer markers and the application of new analytical techniques may help in devising biochemical testing suitable for screening and early diagnosis of malignant diseases.

KEY WORDS: tumor markers, cancer, genetic alterations in cancer, cancer diagnosis, malignant disease.

I. INTRODUCTION

Cancer is a leading cause of mortality and morbidity. The notion exists that early detection through screening may lead to cure or prolongation of life of patients. Clinical biochemists are interested in identifying new biochemical tests for the management of malignant disease. Such tests may be suitable for screening, diagnosis, monitoring, and prognosis. Recently, the area of oncogenes and tumor suppressor genes has reached a state of explosive growth. In this review the possibilities of using oncogenes and tumor suppressor genes as biochemical tests are examined in detail.

Cancer is now considered a genetic disease.[1] Frequently, genes of cancer patients have been found to malfunction. This may be due to gene translocation

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(gene movement to abnormal positions), amplification (acquiring two or more gene copies), mutation (change of normal sequence), deletion (loss of a gene or section of a gene), or abnormal regulation (under- or overexpression). Other mechanisms of malfunction may also exist. Many genetic alterations in cancer involve genes that are called oncogenes or tumor suppressor genes (described in detail below). These are genes that regulate cell growth and differentiation and can be studied with molecular biology techniques. The proteins encoded by these genes are known in most instances, and monoclonal and polyclonal antibodies to both native and mutant proteins are available for immunohistochemical and immunological assays.

Oncogene and tumor suppressor gene testing at the DNA, mRNA, and protein levels will likely become routine in the future to yield information useful for diagnosis, prognosis, monitoring, or treatment. This is already true for some of the currently known oncogenes and tumor suppressor genes. The more likely testing strategy is panel testing at the genetic and protein levels, as cancer is now known to result from multiple genetic abnormalities accumulating over relatively long periods of time.[2] The number of known oncogenes and tumor suppressor genes currently exceeds 50. Thus, comprehensive testing will probably become centralized in specialized oncogene laboratories.

II. ONCOGENES AND TUMOR SUPPRESSOR GENES

Normal cells grow, divide, communicate, and differentiate in a coordinated fashion, all accomplished by the programmed expression of many different genes. It is currently believed that tumor formation arises as a consequence of alterations in genes that are involved in the control of cell proliferation.

By definition, an oncogene is a gene whose abnormal expression or altered gene product leads to malignant transformation.[3] In the healthy state, such genes should not exist in the human genome. Oncogenes represent either normal genes that have been altered or exogenous genetic material.

Normal cellular genes that can give rise to oncogenes are called proto-oncogenes. A proto-oncogene can be transformed to an oncogene by a genetic process called "oncogene activation."

More recently, sets of genes have been found that are negative regulators of cell growth.[4–6] These genes are now collectively called "tumor suppressor genes" or "antioncogenes," but the terms are not precisely descriptive of their functions. It is believed that loss or inactivation of tumor suppressor genes will free cells from negative growth signals and may also lead to malignant transformation. There is, however, an important distinction between the activation process of oncogenes and tumor suppressor genes. Each cell has not one but two genes of any kind. Oncogenes are considered "dominant" genes because they can transform cells despite the simultaneous expression of the other normal allele. An oncogene is thus a promoter of cell growth deregulation, and this promotion is not affected by the presence of the normal allele.

Tumor suppressor genes are considered "recessive" genes because the loss or inactivation of one allele does not, at least in theory, lead to any change in function of the cell. The other normal allele will still produce the physiological product that negatively regulates the growth of the cell.

Whereas the recessive nature of tumor suppressor genes is widely accepted, there are some important exceptions. When a heterozygous tumor suppressor gene locus is involved in carcinogenesis, the mutation is called "dominant negative." Dominant negative mutations have been explained by different mechanisms. For example, the mutant protein may inactivate the wild-type protein through complexation, the wild-type protein may be produced at levels below a threshold level necessary for negative regulation, or the mutant product may acquire oncogenic properties and act like an oncogene.

It was known for many years that acutely transforming retroviruses (ATR) are able to transform cells in culture within days and induce tumors in animals with latency periods of 2 to 8 weeks. Intensive studies with these RNA tumor viruses revealed that they carry specific genes that cause tumorigenesis. These genes were called *v-onc*, standing for viral oncogenes. A major breakthrough occurred in 1976 when it was found that *v-onc* share extensive homology to host genes, the cellular proto-oncogenes.[7]

These observations led to the concept that normal cells contain genes responsible for cell growth and differentiation (proto-oncogenes). These proto-oncogenes can be activated to oncogenes (*c-onc*) by various mechanisms described later in this review. Proto-oncogenes can also be taken up by retroviruses and become transforming oncogenes (*v-onc*) after extensive mutations. The realization that the retroviral oncogenes have cellular homologs led to the identification of many cellular proto-oncogenes by studying the retroviral genes. The genes responsible for the tumorigenic potential of DNA tumor viruses have no apparent relation to cellular genes. Gene products of DNA tumor viruses are also believed to be involved in the control of cell proliferation.

The emerging picture related to the pathogenesis of cancer is that this disease is the outcome of multiple genetic abnormalities occurring and accumulating over long periods. This process includes activation of oncogenes and inactivation of tumor suppressor genes. Oncogene research is now focused on the following objectives:

1. Identification of new oncogenes
2. Study of the differences between proto-oncogenes and oncogenes in order to understand the process of oncogene activation, especially at different stages of the malignant disease
3. The role of proto-oncogene and oncogene products in the control of cell proliferation. The hope is that a byproduct of these studies will be the development of new diagnostic tests based on oncogenes and tumor suppressor genes that will be useful for diagnosis, prognosis, monitoring, and treatment of malignant disease.

III. METHODS FOR THE IDENTIFICATION OF ONCOGENES AND TUMOR SUPPRESSOR GENES

Most of the known proto-oncogenes in the human genome have been identified through the study of the transduced genes of the acutely transforming retroviruses (*v-onc*). These genes can induce tumors in animals and transform cells in culture with very short latency periods (2 to 8 weeks). Another observation related to retroviruses was that in some instances, animals infected with these agents could also develop tumors but with much longer latency periods (many months). Intensive studies have revealed that in these cases, tumorigenesis was not due to retroviral oncogenes per se but to the sites of integration of the retroviral gene into the host gene during viral replication. It was found that these integration sites were close to the loci of proto-oncogenes that then became deregulated. Thus, by studying retroviral integration sites it is possible to identify putative proto-oncogenes.[8]

Nonrandom translocations have been observed in a number of human malignancies.[9,10] For example, in patients with chronic myelogenous leukemia (CML) there is a consistent translocation of a small piece of chromosome 9 to chromosome 22. It was found that as a result of this translocation, a proto-oncogene is positioned adjacent to a normal gene on chromosome 22. This translocation results in the production of a new fusion protein that is thought to be the etiologic agent of CML.

Thus, by studying breakpoints of consistent translocations found in malignancy, it is possible to identify new oncogenes. Other clues for oncogene identification come from studies of gene amplification. In malignant cells, it is frequently found that certain genes are preferentially amplified (an increase in their copy number). In many cases, these turn out to be known oncogenes. By studying gene amplification in malignant tissue, it may be possible to identify new oncogenes.

The methodology that is used to identify putative oncogenes was traditionally based on nucleic acid transfer. In this assay, NIH/3T3 cells (a mouse cell line that grows in a monolayer) are transfected with DNA extracted from tumors. The cells are then examined microscopically for the presence of transformation. This is usually evident by the formation of foci of cells that lose contact inhibition and grow in piles. DNA from the transformed cells is then studied to identify the foreign DNA that caused the transformation.[11]

More recently, oncogenes have been identified by gene cloning and sequencing techniques. The speed by which new genes can be cloned and sequenced has increased dramatically in the last few years. When genes are cloned and found to encode for growth factors or growth factor receptors, these genes are classified as putative proto-oncogenes because many known proto-oncogenes belong to these classes of compounds.

Tumor suppressor genes have been isolated mainly through studies of gene deletions associated with cancer. It is postulated that a consistent loss of genetic material in cancer may be related to the loss of a tumor suppressor gene mapping in that area. For example, in retinoblastoma, there is a loss of genetic material

at the locus 13q14, the site where the retinoblastoma gene was mapped. However, the situation is complex because the loss of one allele is not usually associated with the disease. In many cases, it has been shown that a deletion that deprives the cell of one tumor suppressor gene allele is followed by the loss of the other allele at a later time through either another deletion or a mutation. In some familial cancer syndromes, the loss of one allele might have been inherited. Thus, the individual is born already heterozygous for the tumor suppressor gene locus. More recently, tumor suppressor genes have been mapped by studying polymorphic genetic markers with probes. When it is found that a highly polymorphic marker locus present in normal cells in two distinct copies (heterozygous for both alleles) changes to a homozygous locus in the tumor cells, it is postulated that the marker maps close to a tumor suppressor gene. For more information see Weinberg.[6]

IV. FUNCTIONS OF PROTO-ONCOGENE AND ONCOGENE PRODUCTS

Proto-oncogene products are involved in pathways related to cell division and differentiation. Many proto-oncogenes have been found to be highly conserved through evolution and must play a central role in cell life. Most of the currently known proto-oncogenes and oncogenes have been sequenced, and their respective mRNA and protein products have been characterized. It is now clear that proto-oncogene products are part of the cellular signaling network acting at multiple levels. For example, they act on the cell surface as ligands and growth factors, on the cell membrane as receptors or signal transducers, in the cytoplasm as communication links between the cell membrane and nucleus, and in the nucleus itself where they are involved in gene transcription (Figure 1).[12] Oncogene products presumably affect these same proto-oncogene pathways but in an aberrant fashion. Oncogenes are usually classified on the basis of the function of their products (Table 1).[13] A brief summary of each class is given below.

A. Growth Factors

Comparison of the amino acid sequence of the *v-sis* oncogene revealed high homology with the beta chain of the platelet-derived growth factor (PDGF). PDGF is a serum mitogen required for mesenchymal cell growth in culture. Another oncogene, *hst*, which was identified in a gastric carcinoma, is a member of a family of fibroblast growth factors (FGF) associated with angiogenic properties. Other oncogenes (e.g., *int-1*) are probable growth factors (Table 1). Growth factors are thought to be produced at abnormal levels by tumor cells, and function in an autocrine fashion to stimulate cell proliferation and tumor growth. These mechanisms of action are currently speculative and need to be interpreted with caution, because exogenous addition of PDGF to cells does not cause transformation.

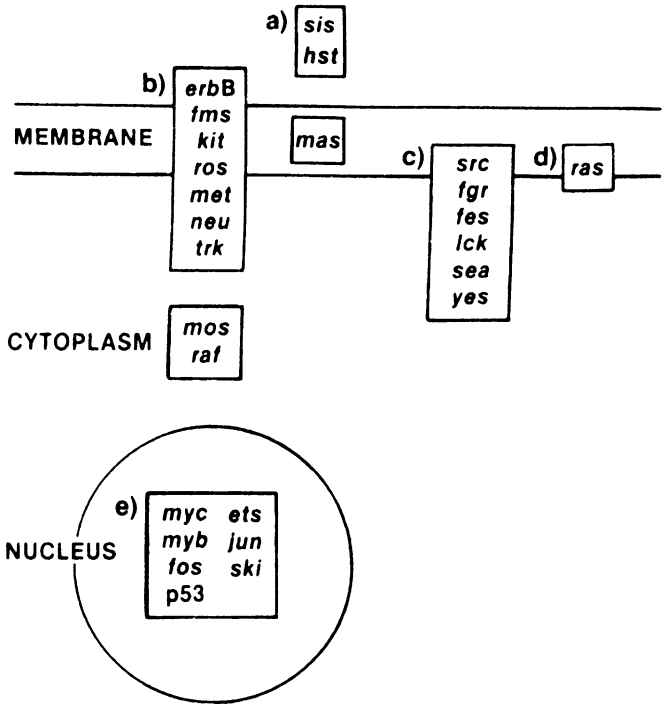

FIGURE 1. Schematic representation of the cellular compartments where oncogene or proto-oncogene products are localized: (a) growth factors; (b) transmembrane tyrosine kinase growth factor receptors; (c and d) membrane-associated proteins of the *src* and *ras* gene family, respectively; (e) oncogenes localized in the nucleus. Two oncogenes localized in the cytoplasm are also shown. (From Park M, Vande Woude GF. *Cancer. Principles and practice of oncology.* Pp. 45–66. Philadelphia: J. B Lippincott, 1989. With permission.)

B. Receptor and Nonreceptor Protein-Tyrosine Kinases

Several dozen protein-tyrosine kinases (PTKs) have now been identified. PTKs are enzymes catalyzing the phosphorylation of tyrosine residues on proteins and are classified into two categories (Figure 2). The first category includes PTKs spanning the plasma membrane, with large extracellular and cytoplasmic domains (receptorlike proteins). An example of this class of molecule is the epidermal growth factor receptor (EGFR). The *v-erbB* oncogene product is a truncated version of EGFR (Figure 2). Other receptorlike PTKs that interact with currently unknown ligands include *trk, met, neu, ros,* and *kit* (Figure 1 and Table 1). Currently, it is believed that oncogenic activation of these proteins by mutation (e.g., compare the physiological EGFR with *v-erbB* or *c-fms* with *v-fms*, Figure 2) or unregulated expression causes unwarranted phosphorylation, uncontrolled enzymatic activity, or disturbance of other circuits of signal transduction, which may initiate oncogenesis. In some cases, it is postulated that these mutated re-

TABLE 1
Functions of Cell-Derived Oncogene Products

	Class 1 — Growth factors
sis	PDGF B-chain growth factor
Int-2	FGF-related growth factor
hst(KS3)	FGF-related growth factor
FGF-5	FGF-related growth factor
int-1	Growth factor?
	Class 2 — Receptor and nonreceptor protein-tyrosine kinases (PTKs)
src	Membrane-associated nonreceptor PTK
yes	Membrane-associated nonreceptor PTK
fgr	Membrane-associated nonreceptor PTK
lck	Membrane-associated nonreceptor PTK
fps/fes	Nonreceptor PTK
abl/bcr-abl	Nonreceptor PTK
ros	Membrane-associated receptor-like PTK
erb	Truncated EGF receptor PTK
neu	Receptorlike PTK
fms	Mutant CSF-1 receptor PTK
met	Soluble truncated receptorlike PTK
trk	Soluble truncated receptorlike PTK
kit (W locus)	Truncated stem-cell receptor PTK
sea	Membrane-associated truncated receptorlike PTK
ret	Truncated receptorlike PTK
	Class 3 — Receptors lacking protein kinase activity
mas	Angiotensin receptor
	Class 4 — Membrane-associated G proteins
H-ras	Membrane-associated GTP-binding/GTPase
K-ras	Membrane-associated GTP-binding/GTPase
N-ras	Membrane-associated GTP-binding/GTPase
gsp	Mutant-activated form of $G_{s\alpha}$
gip	Mutant-activated form of $G_{s\alpha}$
	Class 5 — Cytoplasmic protein-serine kinases
raf/mil	Cytoplasmic protein-serine kinase
pim-1	Cytoplasmic protein-serine kinase
mos	Cytoplasmic protein-serine kinase (cytostatic factor)
cot	Cytoplasmic protein-serine kinase?
	Class 6 — Cytoplasmic regulators
crk	SH-2/3 protein that binds to (and regulates?) phosphotyrosine-containing proteins

TABLE 1 (continued)
Functions of Cell-Derived Oncogene Products

Class 7 — Nuclear transcription factors

myc	Sequence-specific DNA-binding protein
N-myc	Sequence-specific DNA-binding protein?
L-myc	Sequence-specific DNA-binding protein?
myb	Sequence-specific DNA-binding protein
lyl-1	Sequence-specific DNA-binding protein?
p53	Mutant form may sequester wild-type *p53* growth suppressor
fos	Combines with *c-jun* product to form AP-1 transcription factor
jun	Sequence-specific DNA-binding protein; part of AP-1
erbA	Dominant negative mutant thyroxine (T_3) receptor
rel	Dominant negative mutant NF-κB-related protein
vav	Transcription factor?
ets	Sequence-specific DNA-binding protein
ski	Transcription factor?
evi-1	Transcription factor?
gli-1	Transcription factor?
maf	Transcription factor?
pbx	Chimeric E2A-homeobox transcription factor
Hox2.4	Transcription factor?

Unclassified

dbl	Cytoplasmic truncated cytoskeletal protein?
bcl-2	Plasma membrane signal transducer?

Note: The table is selective. These oncogenes were originally detected as retroviral oncogenes or tumor oncogenes. Others were identified at the boundaries of chromosomal translocations and at sites of retroviral insertions in tumors, or were found as amplified genes in tumors and shown to have transforming activity.

From Hunter T, *Cell* 1991; **64:** 249–70. With permission.

ceptors transmit mitogenic signals even though they are not bound to their ligands. This constitutive, unregulated activation may be critical for the oncogenic transformation of cells.

The second category of PTKs is those present in the cytoplasm and attached to the cell membrane through myristilated N-terminal glycine residues. The prototype of these PTKs is *src* (Figure 2). Other members are shown in Table 1. All of these PTKs (including the first category described above) have a catalytic domain that transfers phosphate from ATP to tyrosine residues during either autophosphorylation (i.e., *src* phosphorylates itself) or phosphorylation of target molecules. It is believed that the tumorigenic potential of these PTKs arises from mutations that result in unregulated tyrosine phosphorylation. Transforming PTKs of this class lose their tumorigenic potential if they are not attached to the cell membrane. The *abl* and *fps* oncogene products (the former are discussed in detail later in this review) are classified in this category of PTKs, but they are not

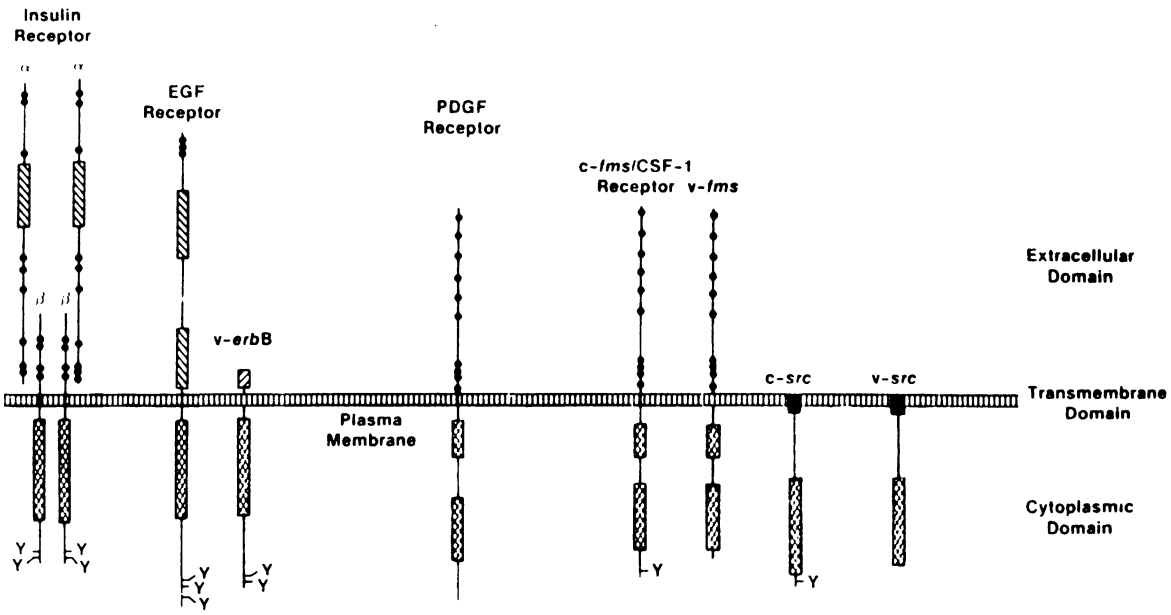

FIGURE 2. Schematic comparison of structural features of cell surface receptors and tyrosine kinase oncogene products. Regions of high cysteine concentration are shown as hatched boxes and single cysteine residues as filled circles. The tyrosine kinase domains are represented as cross-hatched boxes, and the position of carboxyterminal tyrosine residues is shown as Y. The deletions that activate *v-erb, v-fms,* and *v-src* are illustrated by comparing to EGF receptor, *c-fms* and *c-src*, respectively. EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CSF-1, mononuclear phagocyte colony stimulating factor. (From Park M, Vande Woude GF. *Cancer. Principles and practice of oncology. Pp. 45–66. Philadelphia: J. B. Lippincott, 1989. With permission.)*

exclusively membrane associated; part of the *abl* population of molecules is found in the nucleus.

C. Receptors Lacking Protein Kinase Activity

This class consists of only two members. The *mas* oncoprotein is a transmembrane receptor for angiotensin. The other oncoprotein is the serotonin receptor. These gene products can transform even in the unmutated form, presumably through binding of ligands present in the circulation.

D. Membrane-Associated G Proteins

These proteins, which are associated with the cytoplasmic surface of the plasma membrane (Figure 1), include the *ras* gene family members (*H-ras, K-ras, N-ras*) and the G proteins, which are effectors of adenylate cyclase. The *ras* proteins exhibit significant sequence homology with the α-subunit of G pro-

teins such as the Gs, a protein that activates adenylate cyclase in response to β-adrenergic stimuli, and Gi, a protein that inhibits this enzyme and perhaps activates phospholipase C. There is also homology of *ras* with Co, a protein of as yet unknown function, and with transducin, a protein that regulates GMP phosphodiesterase activity in visual signal transduction.

The G proteins are functionally regulated by the binding of guanine nucleotides after receiving signals from specific receptors. The regulatory effect is mediated when the protein exchanges GDP for GTP. This regulation is transient because G proteins, in addition to their GDP- and GTP-binding properties, also exhibit GTPase activity which hydrolyzes GTP to GDP (see below).

The biochemical function of the *ras* proteins, which all have a molecular weight of 21,000 Da (p21), is related to their ability to bind GTP/GDP and to hydrolyze GTP (Figure 3).[14] The p21, in its inactive state, binds GDP. Upon stimulation, probably through a receptor-mediated event, p21 exchanges GDP for GTP and becomes active.

Activated p21 then interacts with effector molecules that are still unidentified to stimulate cell growth. The active state of p21 is transient due to its GTPase activity, which hydrolyzes GTP to GDP. A simplified explanation for the trans-

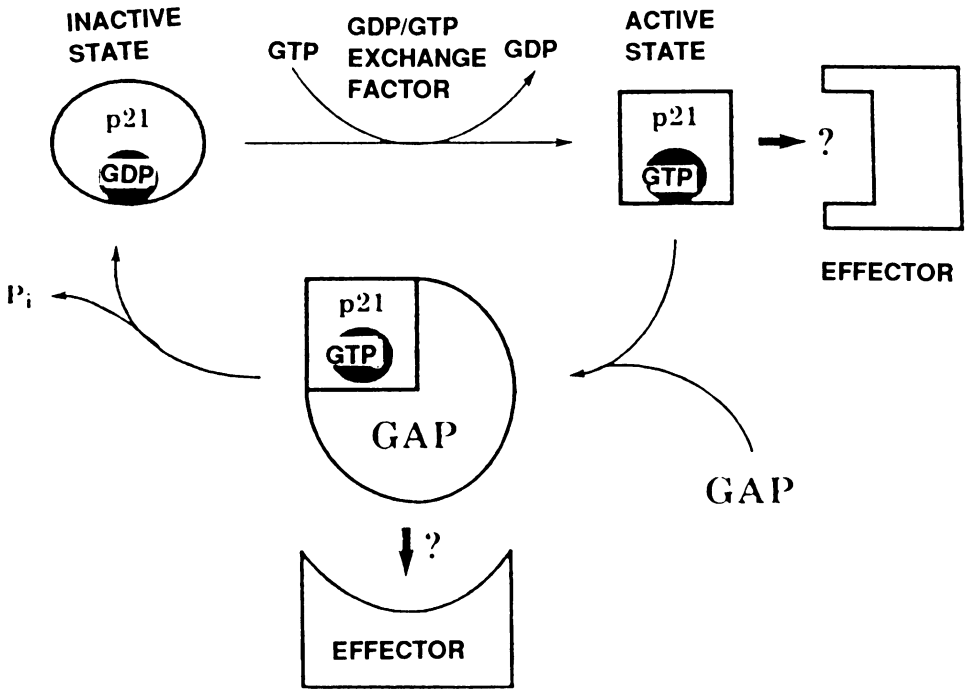

FIGURE 3. Mechanism of activation-deactivation of mammalian *ras* p21 proteins. Inactive *ras* binds GDP but becomes activated when it exchanges GDP for GTP. Activated *ras* interacts with as yet unidentified effector molecules. Activated *ras* is rapidly inactivated through its own GTPase activity or through interaction with the GTPase activating proteins (GAP), a family of compounds that stimulate its GTPase activity. (From Barbacid M, *Eur J Clin Invest,* 1990; **20:** 225–235. With permission.)

forming activity of mutant p21 *ras* would be that mutants have decreased GTPase activity, thus leaving the protein in an activated state for prolonged periods. The low GTPase activity of mutants has been confirmed.

The regulation of this signal transduction system is complex. Recently, proteins have been described, called GTPase-activating proteins, or GAPs, which stimulate the GTPase activity of *ras*.[15] GAPs bind the activated ras and inactivate it by enhancing its GTPase activity (Figure 3). It appears then that GAPs are regulators of *ras*. More complex interactions of signal-transducing molecules are now being discovered. For example, some PTKs can phosphorylate GAP, whereas other effectors like protein kinase C and tumor suppressor genes can interact with GAP as well.[16–18]

E. Cytoplasmic Protein-Serine/Threonine Kinases

These protein kinases phosphorylate serine and threonine amino acids and are soluble cytoplasmic proteins (Table 1 and Figure 1). In addition to the proteins shown in Table 1, another two kinases, protein kinase C and *cdc 2*, are also classified in this category. The product of *mos* is a cytostatic factor that arrests the meiotic cycle in the metaphase.

The mechanism by which *mos* is activated and induces transformation is unknown; it is speculated that increased phosphorylation of targets results in the loss of cell cycle control. The *raf* protein enzymatic activity is induced by mitogens; the protein also interacts with activated PDGF and EGF receptors. These findings and the observation that stimulated *raf* moves to the nucleus, where it may phosphorylate transcription factors, supports the view that *raf* acts as a transporter of signals from the cell membrane to the nucleus.[19] Oncogenic forms of *raf* are thought by some to be mutants that have lost regulatory sequences and thus are permanently active. Others believe that mutant *raf* kinase is relocated and disturbs nonphysiological substrates.

The tumorigenic potential of protein kinase C in human cancers has not yet been demonstrated. The *cdc 2* kinase seems to play an important role in cell cycle control. Substrates for this kinase are p53 (see later in this review), PTKs of the *src* family, and *abl*. However, it is not yet clear if *cdc 2* can induce tumorigenesis. Little is known about *pim-1* and *cot* proteins.

F. Cytoplasmic Regulators

The only member of this class is the *v-crk*, which contains in its structure regulatory sequences called SH-2 and SH-3 sequences. These sequences are also found in the PTKs *src*, *fps*, and *abl*, as well as in other proteins. However, *v-crk* lacks PTK activity.

It is postulated that *v-crk* binds to a specific phosphotyrosine of *src* that renders src active. If this binding does not happen, the *src* phosphotyrosine will

bind to SH-2 present on *src* itself and inactivate the kinase activity of the molecule. Thus, *v-crk* binding to *src* phosphotyrosine will leave *src* active. This may cause unwarranted phosphorylation and possibly transformation.[20]

G. Nuclear Transcription Factors

Many of the currently known proto-oncogene products are found in the cell nucleus (Figure 1 and Table 1). The majority of these proteins exhibit sequence-specific DNA-binding ability, or they are putative transcription factors. They are considered regulators of gene expression. Some of these proteins are expressed at a precise segment of the cell cycle in a well-orchestrated temporal sequence. This programmed expression is important for facilitating the passage of the cell from G_0 to G_1 and S phases.

The loss of the normal function of these nuclear proteins can cause malignant transformation, but the mechanisms have not yet been elucidated. It is thought that mutant transcription factors may induce the expression of genes that are inappropriate. Another scenario would be that mutant transcription factors inhibit the expression of genes that are needed for differentiation. Regardless of the mechanism, the disturbed gene expression can trigger tumorigenesis through deregulation of cell growth and differentiation.

V. FUNCTIONS OF TUMOR SUPPRESSOR GENES

The best-studied tumor suppressor genes are listed in Table 2. More extensive lists of putative tumor suppressor genes have also been published.[6] The presence of these genes was initially suggested from cell fusion experiments that revealed that hybrids of normal and tumor cells are generally nontumorigenic. These data suggested that normal cells, but not their tumorigenic counterparts, contain genes that can suppress the tumorigenic phenotype.

The mechanisms whereby tumor suppressor genes function are not yet clear. These genes, when mutated or deleted, deprive the cell of their protein products. It is reasonable to postulate that these proteins are repressors of biochemical functions and cellular proliferation. The *Rb* and *p53* gene products bind and inactivate the large T antigen, a transforming protein of the virus SV40. This observation led to the hypothesis that *Rb* and *p53* may bind to endogenous ligands involved in the promotion of cell proliferation and especially in the control of the cell cycle.[21] Mutations or deletions of *Rb* or *p53* can abolish this binding and the suppression effects. This theory is in accord with the observation that mutant *Rb* of *p53* cannot bind the SV40 large T antigen. The NF-1 gene encodes a protein that may negatively regulate *ras* proteins, an observation that suggests that tumor suppressor genes act as anti-oncogenes by nullifying the actions of oncogenes.[22]

TABLE 2
Tumor Suppressor Genes

Gene	Chromosomal location	Gene inactivated in tumor type	Protein
Rb	13q	Retinoblastoma, osteosarcoma, SCLC, breast, colon, stomach, bladder	105-kDa nuclear phosphoprotein; transcription factor?
p53	17p	Leukemia, bladder, liver, brain, lung, colon, and others	53-kDa nuclear phosphoprotein; transcription factor?
DCC	18q	Colon carcinoma	190-kDa transmembrane phosphoprotein; cell receptor involved in signal transduction?
NF-1	17q	Neurofibromatosis type 1	250-kDa protein having homology to GTPase activating proteins (GAP); down regulator of *ras*?
WT	11p	Wilm's tumor, breast, lung, bladder, rhabdomyosarcoma	35-kDa protein having four zinc finger domains that bind DNA; transcription factor?

Note:
p short arm of chromosome
q long arm of chromosome
SCLC small-cell lung carcinoma
DCC deleted-in-colon carcinoma
NF neurofibromatosis
WT Wilm's tumor

The *DCC* gene may encode a receptor that is involved in signal transduction.[23] As *Rb*, *p53*, and *WT* gene products are nuclear proteins with DNA-binding ability, it is suggested that these proteins may also act as transcription factors.

VI. MULTIPLE STEPS IN TUMORIGENESIS

It is now widely accepted that tumorigenesis is a multistep process that involves not one but multiple genetic alterations.[2] Although evidence is circumstantial, the current belief is that the multiple genetic abnormalities in cancer are pathogenetic factors and not merely epiphenomena of the malignant process. These steps include oncogene activations, losses of tumor suppressor genes, DNA hypomethylation, and changes in the levels of gene expression (deregulation). These multiple changes usually occur in a preferred sequence (Figure 4). However, it is now believed that the total accumulation of events, irrespective of

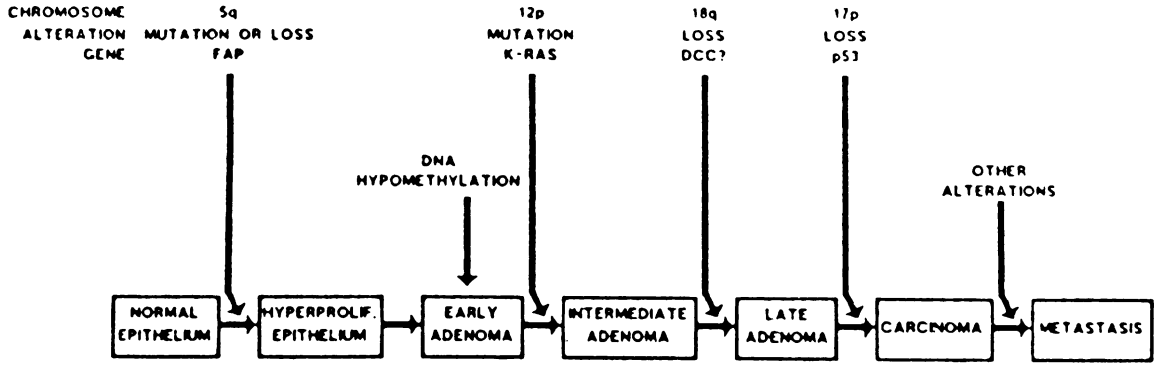

FIGURE 4. A genetic model for colorectal tumorigenesis. Tumorigenesis proceeds through a series of genetic alterations involving oncogenes (12p mutation of *K-ras*, written as *ras* in other parts of this review) and tumor suppressor genes, particularly those on chromosomes 5q (locus of the familial adenomatous polyposis gene, FAP), 17p (p53 locus), and 18q (locus of the deleted-in-colon carcinoma gene, DCC). The three stages of adenomas represent tumors of increasing size, dysplasia, and villous content. p and q represent the short and long arms of the mentioned chromosomes. (From Fearon ER, Vogelstein B. *Cell,* 1990; **61:** 759–67. With permission.)

sequence, is also a major contributor to malignancy. These findings suggest that multiple testing with batteries of biochemical indicators will be the preferred strategy to study malignant disease. Early alterations should be more advantageous for screening than alterations occurring very late or after initiation of malignancy.

VII. MECHANISMS OF ONCOGENE ACTIVATION

Oncogene activation is the process that converts proto-oncogenes to oncogenes. Oncogene activation is associated with a genetic alteration that can vary from subtle to gross. For example, it may be a single-base change in a gene, or it may involve many changes in bases, large deletions, or translocations. The result of the oncogene activation is either unscheduled expression or unregulated increase of the normal product, or expression of an altered, mutated product with tumorigenic activity.

The most important known mechanisms of oncogene activation are described briefly below. The understanding of these mechanisms is important in the design of diagnostic strategies.

A. Oncogenes of Acutely Transforming Retroviruses

The oncogenes of acutely transforming retroviruses (*v-onc*) have extensive homology to cellular proto-oncogenes. Because *v-onc* are tumorigenic, examination of the differences between *v-onc* and cellular proto-oncogenes can offer clues to mechanisms of oncogene activation. Some of the differences include

multiple point mutations and deletions of upstream or downstream exons or of transcriptional and posttranscriptional regulatory elements. Some examples of differences between *v-src* and *c-src* and *v-fms* and *c-fms* are shown in Figure 2. Deletions at the carboxyterminal region are crucial for activation of both proto-oncogenes.

Although these comparisons between *v-onc* and proto-oncogenes are useful for understanding the mechanisms of oncogene activation, the differences found are not necessarily prevalent in human cancers. Currently, these differences do not offer possibilities for practical biochemical testing related to diagnosis of malignant disease.

B. Oncogene Activation by Insertional Mutagenesis

Foreign DNA can be inserted, causing interruption of normal genes (structural damage) or modification of regulatory sequences (regulatory damage). Altered gene products may result that could be biochemically hyperactive or hypoactive, or normal gene products may be formed in inappropriate amounts or at the wrong time. In erythroleukemias or lymphomas induced by avian leukosis virus, and in mammary tumors induced by viruses in mice, the activated oncogene contains inserted DNA. This mechanism is useful in understanding the origin of some animal tumors and in the isolation of critical gene sequences, but it is not prevalent in human malignancies and currently offers no possibilities for the design of diagnostic tests.

C. Chromosomal Rearrangements and Oncogene Activation

Movement of genetic material from its normal position to another (translocation) has been consistently observed in some human malignancies (Table 3). In 80% of Burkitt's lymphoma patients, the *c-myc* locus on chromosome 8 (8q24) is translocated to a locus on chromosome 14 (14q32). This translocation repositions *c-myc* distally but close to the immunoglobulin heavy-chain gene locus. In the remaining 20% of patients, the *c-myc* gene remains on chromosome 8, but the loci of the k-light chain gene (5% of cases) or λ-light chain gene (15% of cases) translocate from chromosomes 2 and 22, respectively, to a locus adjacent to *c-myc* on chromosome 8. The involvement of *c-myc* in the pathogenesis of Burkitt's lymphoma is very likely, although the exact mechanism f *c-myc* activation due to the translocations is not fully understood. The current consensus is that the translocation triggers the constitutive, uncontrolled expression of *c-myc*, which gives rise to the lymphoma.

In at least 90% of patients with chronic myelogenous leukemia (CML) and in 10 to 15% of patients with acute lymphocytic leukemia (ALL), the Philadelphia chromosome (Ph) is found in the karyotype of malignant cells. Ph is formed from a translocation of genetic material between chromosomes 9 and 22, which causes

TABLE 3
Chromosomal Translocations in Human Malignancies

Gene locus	Human neoplasm	Percentage of tumors with translocation of gene rearrangement	Chromosome translocation
c-myc	Burkitt's lymphoma	80	t(8;14)(q24;q32)
		15	t(8;22)(q34;q11)
		5	t(2;8)(q11;q24)
bcr-abl	Chronic myelogenous leukemia	90–95	t(9;22)(q34;q11)
	Acute lymphocytic leukemia	10–15	t(9;22)(q34;q11)
bcl-1	Chronic lymphocytic leukemia of B cell type	10–20	t(11;14)(q13;q32)
bcl-2	Follicular lymphoma	85–95	t(14;18)(q32;q21)

From Park M, Vande Woude GF. *Cancer. Principles and practice of oncology.* Pp. 45–66. Philadelphia: J. B. Lippincott, 1989. With permission.

chromosome 22 to become shorter (this is the Ph chromosome) and chromosome 9 to become longer.[10]

The translocation brings the proto-oncogene *c-abl* from its normal position on chromosome 9 to an area on chromosome 22 that is called *bcr* (breakpoint cluster region). The juxtaposed *bcr* and *abl* genes produce an 8.5-kp *bcr-abl* hybrid mRNA that encodes a novel protein having a molecular weight of 210 kDa. This protein is considered the etiologic factor of CML. Diagnostic tests for the Ph-positive CML (and ALL, see later in this review) are based on either DNA rearrangement studies at the *bcr* locus, detection of the 8.5-kb *bcr-abl* mRNA, or detection of the 210-kDa protein.

D. Gene Amplification

Cytogenetically visible differences between normal and cancer cells, known as double minutes (DM) and homogeneously staining regions (HSR) were known for many years. It is now known that DM and HSR contain multiple copies of genes (amplified genes). Gene amplification is a usual finding in cell lines derived from tumors. The amplified genes are usually oncogenes (Table 4). Gene amplification is usually a predictor of poor clinical prognosis. It is postulated that the increased number of gene copies causes increased levels of mRNA and protein, which can induce the malignant transformation. In many cases, the sequences of normal and amplified genes have been determined. No differences were present,

TABLE 4
Cellular Oncogenes Amplified in Human Tumors

Tumor	Oncogene	Amplification
Small-cell lung cancer	*c-myc*	Up to 80X
	N-myc	Up to 50X
	L-myc	Up to 20X
Neuroblastoma	*N-myc*	Up to 250X
Glioblastoma	*c-erB* (EGFR)	Up to 50X
Mammary carcinoma	*c-erB-2* (HER2)	Up to 30X

From Park M, Vande Woude GF. *Cancer. Principles and practice of oncology.* Pp. 45–66. Philadelphia: J. B. Lippincott, 1989. With permission.

confirming that the oncogene activation in these cases is associated with overproduction of a normal protein.

E. Oncogene Activation by Point Mutation

A single point mutation at specific codons of the *ras* gene family is enough to activate this oncogene. Studies have shown that such mutations can markedly decrease the GTPase activity of *ras*, which is then left in the activated form as shown in Figure 3. Specific examples of *ras* gene mutations in human cancers are given later in this review.

VIII. APPLICATIONS OF ONCOGENES AND TUMOR SUPPRESSOR GENES IN HUMAN DISEASE

Oncogenes and tumor suppressor genes can be studied at three different levels: DNA, mRNA, and protein, providing the potential of new ways for the diagnosis, monitoring, and treatment of the malignant disease. For example, an altered gene product may be released into blood, urine, or feces, and its detection may alert the physician to the presence of a tumor. Alternatively, these altered gene products may be immunogenic, and circulating antibodies may signal the presence of the disease. These two possibilities represent the most attractive diagnostic modalities. Approaches involving studies of tumor tissue are useful only for prognosis and classification.

In the vast majority of patients with cancer, the genetic abnormalities observed are "somatic," which means that they are present only in tumor cells and not in normal or germline cells. These genetic abnormalities cannot be transmitted to offspring. Inherited forms of cancer, however, do exist, and in these cases the

genetic abnormalities are present in germline cells. Here, the testing of DNA may be very useful in identifying genetic predisposition, e.g., heterozygotes for a tumor suppressor gene. Such individuals may then be monitored closely and given preventive therapy or early therapy for cancer once it has developed.

Identification of genetic changes in cancer cells may be used for prognosis. For example, gene amplification was found to be an unfavorable prognostic finding in breast carcinoma and neuroblastoma. Genetic changes may also be useful in differential diagnosis, in classification, and in selection of therapeutic interventions.

The genetic abnormalities seen in oncogenes and tumor suppressor genes are considered causative for cancer rather than being late sequelae or epiphenomena, and it is hoped that definition of these genetic changes at the DNA, mRNA, or protein levels, may lead to successful treatment.[24] For example, defective protein products of oncogenes like *ras* and *erbB2* can be targeted with antibodies or specific inhibitors. Other developments include attempts to restore the lost tumor suppressor genes by gene transfer techniques, with encouraging results *in vitro*.[25] Table 5 lists some applications of oncogenes in clinical medicine.[1]

A. Clinical Applications of the *bcr-abl* Translocation

More than 90% of patients with CML carry the Ph chromosome in the leukemic cell population. The translocation brings most of the *c-abl* proto-oncogene from chromosome 9 to the *bcr* locus on chromosome 22.[26] Expression of the fused gene gives rise to an 8.5-kb chimeric mRNA, which in turn is translated to a 210-kDa protein (p210).[27–34] Variants producing fusion mRNAs of a different size also exist (see below). The normal *c-abl* proto-oncogene is transcribed into a 6.0 and 7.0-kb mRNA, which gives rise to a 150-kDa protein (p150). About 15% of patients with ALL also carry the Ph chromosome. In ALL, however, the fusion mRNA is shorter (7.0 kb) and gives rise to a 190-kDa protein (p190). The major fusion mRNAs consisting of exons of both the *bcr* and the *abl* loci are described below.

Diagnostic tests for CML and ALL are based on the fusion of *bcr* and *abl* loci and can be performed at the level of DNA, mRNA, or protein. Each of these tests has merits and limitations. Here, the sensitivity of the test is of primary importance because it is used in patients who have undergone bone marrow transplantation or patients in remission or early stages of relapse. In these cases, the percentage of leukemic cells may be very low.

At the DNA level, the translocation can be studied with probes hybridizing inside the breakpoint region of chromosome 22. This region is approximately 5.8 kb long. When the translocated gene from chromosome 9 is inserted, changes in restriction enzyme sites create new DNA fragments hybridizing with the probe.[35] These new bands (rearrangements) can be seen on Southern blots along with the normal bands from the unaffected chromosome (Figure 5).

TABLE 5
Oncogenes at the Bedside

Tumor	Locus	Applicable feature	Application[a]
Acute lymphocytic leukemia	*bcr-abl*	Distinctive translocation	Distinguish from chronic myelogenous leukemia
Adenocarcinoma of lung	*K-ras*	Point mutations	Prognosis
Chronic myelogenous leukemia	*bcr-abl*	Translocation breakpoint detectable	Diagnosis in absence of Philadelphia chromosome
Carcinoma of breast	*erbB-1*	Overexpression	Prognosis
	neu	Amplficiation	Prognosis
	11q13	Amplification	Prognosis
	11p	Deletion	Prognosis
	myc	Amplification	Prognosis
Myelodysplasia	*N-ras/K-ras*	Point mutations	Prognosis
Neuroblastoma	*N-myc*	Amplification	Prognosis and selection of therapy
Retinoblastoma	*Rb1*	Loss or damage	Detection of predisposition

[a] The list offers representative examples of varying certainty and prospect.

From Bishop JM. *Cell* 1991; **64:** 235–48. With permission.

Although the breakpoint regions on chromosomes 9 and 22 are variable, they occur only in introns and therefore produce only a small number of mature mRNAs. These are shown in Figure 6; they contain junctions between exon II of *abl* and either exon 3, exon 2, or the 5′-exon of *bcr*. These mRNAs give rise to proteins of varying molecular weight, between 210 and 190 kDa; the normal *abl* encodes a protein of 140 to 150 kDa. Normal and abnormal proteins can be quantified by using metabolic labeling with ^{32}P-phosphate followed by immunoprecipitation with antibodies, agarose gel electrophoresis, and autoradiography (Figure 7).

Alternatively, labeling with ^{32}P can be achieved by using the kinase activity of the protein and ^{32}P-ATP. The availability of specific antibodies against the various protein products of the fusion genes can also permit the assays of the proteins using immunometric assay principles.

The analysis of specific mRNAs can also be used as a diagnostic test for CML and AML. Northern blot analysis reveals the abnormal mRNAs in CML (8.5 kb) and ALL (7.4 kb) (Figure 8). However, more elaborate procedures for detecting the aberrant forms of mRNA in CML and ALL have been described, based on PCR amplification.[36] The strategy is shown in Figure 6. The mRNAs are reverse-transcribed with reverse transcriptase to the corresponding cDNAs. They are then amplified with PCR using appropriate primers. Primer A binds to

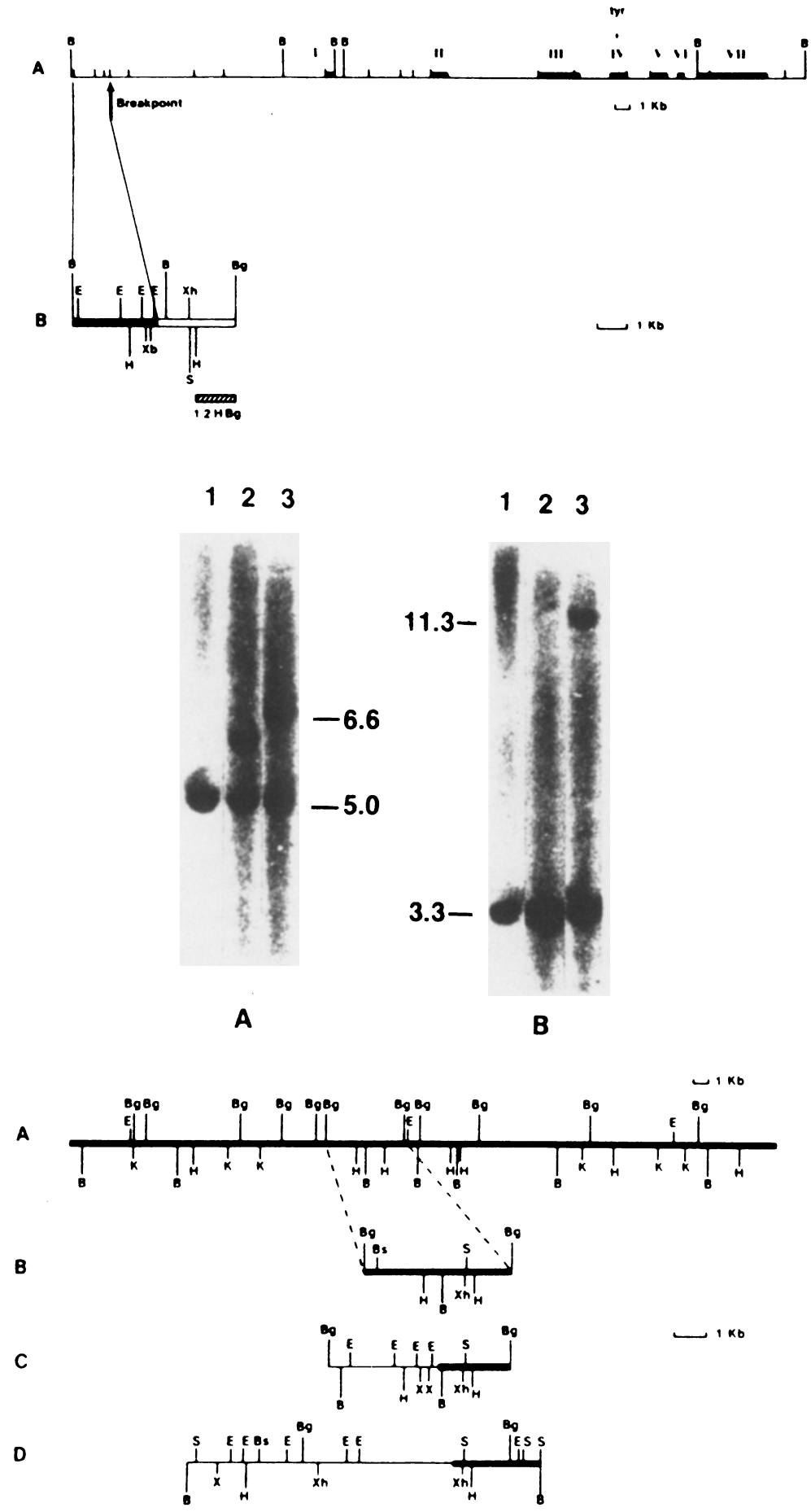

FIGURE 5. Restriction fragment length polymorphism analysis of normal DNA (middle

panel, lane 1) and DNA from two patients with chronic myelogenous leukemia, CML (lanes 2 and 3). These patients are coded as 0319129 and 02120185 in the original publication.[35] The normal DNA was from a human cell line coded GM3314. Upper panel: A region of chromosome 9 containing the *c-abl* proto-oncogene. Note that in CML, a piece of DNA from this chromosome moves to a region on chromosome 22 (translocation). This is shown in B, where the solid bar indicates sequences from chromosome 9 and the open bar represents sequences from chromosome 22. The area of DNA that contains the junction was mapped with the various restriction enzymes shown. B = BamHI; Bg = BglII; E = EcoRI; H = HindIII; S = SstI; Xb = XbaI; Xh = XhoI. For the polymorphism analysis, a labeled probe was used, shown as a hatched box. This probe, coded HBg, is 1.2 kb long and hybridizes to sequences on chromosome 22, between restriction enzyme sites H and Bg as shown. Middle panel: Restriction analysis of DNA. Ten micrograms of high-molecular-weight DNA were digested with either BglII (left) or BamHI (right), electrophoresed on 0.75% agarose gels, and Southern-blotted. After hybridization with radiolabeled HBg probe and autoradiography, the results were obtained. Note that with BglII digestion, a band 5.0 kb long is obtained from the normal DNA (lane 1). With CML DNA, the normal band is also seen, but there are additional bands at 6.0 kb (lane 2, patient 0319129) and 6.6 kb (lane 3, patient 02120185). Similarly, bands at 3.3 kb (normal band) and 11.3 kb (patient 02120185) are seen when the DNA was digested with BamHI. The appearance of the abnormal bands is due to the translocation, as explained further in the lower panel, and can be used for the diagnosis of CML. Lower panel: (A) Part of chromosome 22 in which chromosomal breaks occur and restriction enzyme map. Additional enzymes are Bs = BstEII and K = KpnI. The chromosomal part shown in B, about 5-kb long, is created by restriction enzyme BglII and is the piece detected by the probe in lanes 1, 2 and 3 of middle left panel. A close examination in that area reveals the two BamHI. (B) sites that give rise to the 3.3-kb fragment in the middle right panel. (C) After translocation in patient 0319129, part of chromosome 9 (thin line) was attached to chromosome 22 (heavy line) in the region between the two BglII sites. Note that due to the translocation, one BglII site from panel B (the left one) was lost and another BglII site, lying on DNA from chromosome 9, appeared. The distance between the two BglII sites is now 6.0 kb, and it appears as a band in lane 2, middle left panel. Note that the BamHI sites in chromosome 22 were not affected, and no extra band appears in lane 2 of middle right panel. (D) After translocation in patient 02120185, the hybrid DNA is shown, with restriction enzyme sites. Note that in this patient, the distance between BglII sites is 6.6 kb, giving rise to the band shown in lane 3, middle left panel. Also note that one BamHI site on chromosome 22 is affected (eliminated), and a new one appears on the DNA from chromosome 9 but at a distance of 11.3 kb, giving rise to the band in lane 3, middle right panel. (From Grotten J, Stephenson JR, Heistenkamp N, et al. *Cell,* 1984; **36:** 93–99. With permission.)

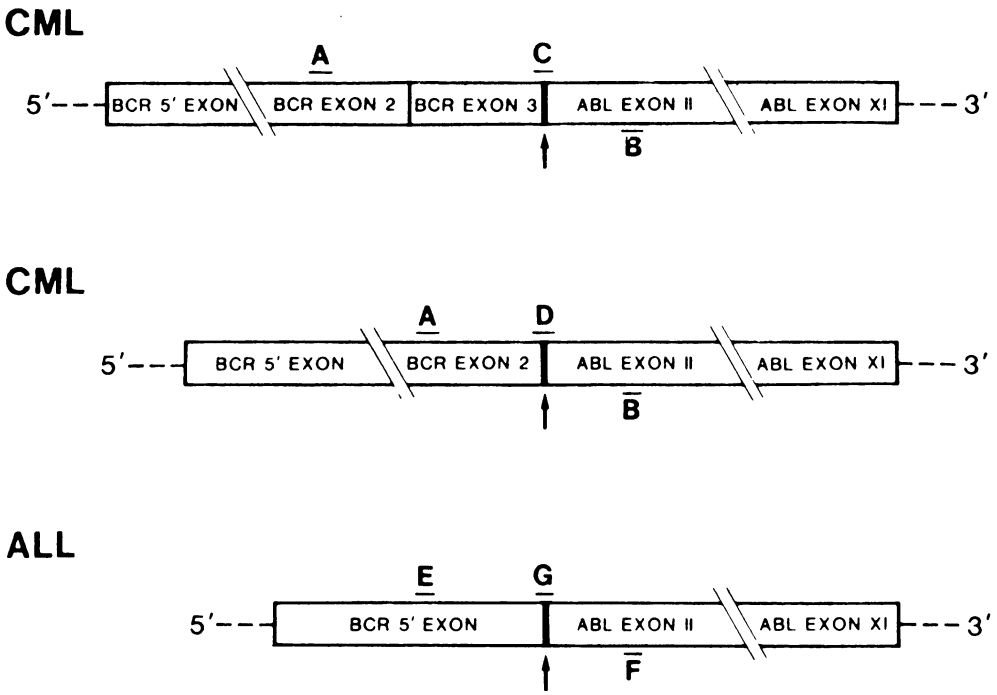

FIGURE 6. Structures of chimeric mRNAs in patients with chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL). Exons of the *bcr* region belong to sequences on chromosome 22, and *abl* exons belong to sequences on chromosome 9. The junction sites are shown in points C, D, and G. Using PCR and the two shown primers A and B, the *bcr-abl* junction sequences found in CML can be amplified. The PCR product can then be detected by Southern blot analysis, using probes hybridizing in the junction regions C and D. Similarly, using primers E and F, the junction sequence found in ALL can be amplified and detected with probe G. The arrows point to the junction between BCR and ABL exons (written as *bcr* and *abl* in the text). The results of such analyses, which are diagnostic for CML and ALL, are presented in Figure 9. (From Kawasaki ES, Clark SS, Coyne MY, et al. *Proc Natl Acad Sci USA* 1988; **85:** 5698–5702. With permission.)

sequences on exon 2 of *bcr*; primer E binds to sequences on the 5′ exon of *bcr*. Similarly, primers B and F bind to sequences on exon II of *abl*. PCR will yield fragments that are 200, 125, or 307 bases long, respectively. The fragments are then run on agarose gel electrophoresis, Southern-transferred, and hybridized to probes, C, D, or G; these probes recognize only the junction sequences of *bcr* and *abl*, as shown in Figure 6. The method is thus highly specific only for the chimeric mRNAs. Some typical results are shown in Figure 9. The aberrant mRNAs are easily detected.

The various possible strategies for the diagnosis of CML or ALL at the DNA, mRNA, or protein level are simple. Which method is the most advantageous? Methods based on direct assays of DNA or mRNA (Southern or Northern analysis) are straightforward. However, the PCR amplification step used in the study of mRNAs gives this method the extreme sensitivity needed for patients who have undergone bone marrow transplantation or patients who are in remission or early

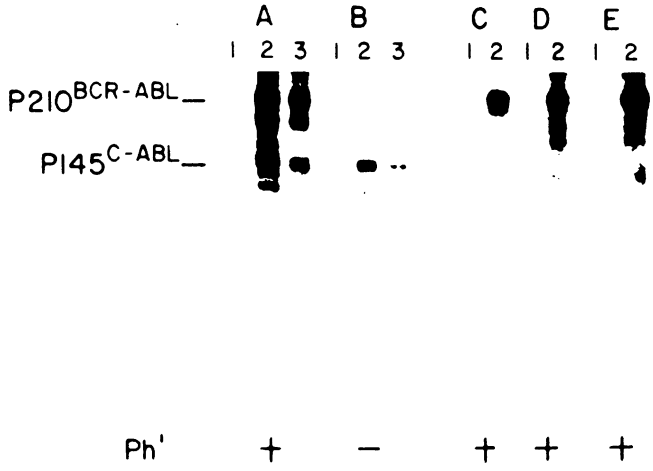

FIGURE 7. Diagnosis of chronic myelogenous leukemia (CML) by analyzing samples for the presence of the abnormal 210-kDa chimeric protein (P240 BCR-ABL) or the 145-kDa normal protein product of *c-abl* (P145 C-ABL). The loadings were (A) positive CML control (cell line K562); (B) patient 1 (normal); (C) positive CML control as in A; (D) patient 2 (positive); (E) patient 3 (positive). The data in A and B were obtained as follows: cells K562 or from patient 1 were metabolically loaded with ^{32}P-phosphate and lysed. The proteins P210 and P145 were then immunoprecipitated with specific antisera (lane 1, nonimmune serum; lane 2, rabbit anti-pEX5 antibody; lane 3, rabbit anti-pEX2 antibody). The immunoprecipitated proteins were then separated by sodium dodecyl sulfate-PAGE and detected by autoradiography. Note the presence of the abnormal and normal protein bands at 210 and 145 kDa, respectively, in the positive control (A) and the presence of only the normal protein in patient 1 (B) who is Philadelphia-chromosome-negative. The analysis of data in C, D, and E is the same, but the labeling with ^{32}P was done by using the kinase activity of the 210-kDa protein. In this case, the radioactive substrate is ^{32}P-ATP. Note the abnormal bands at 210 kDa in cases C, D, and E, which have the Philadelphia chromosome. (From Kawasaki ES, Clark SS, Coyne MY, et al. *Proc Natl Acad Sci USA* 1988; **85:** 5698–5702. With permission.)

stages of the disease. The PCR method can probably detect aberrant mRNA from one leukemic cell. This compares to 10^4 cells needed to detect the aberrant protein product. Because both the mRNAs and the proteins originating from the fusion of *bcr* and *abl* are unique substances not found in any normal state, they are "true tumor markers."

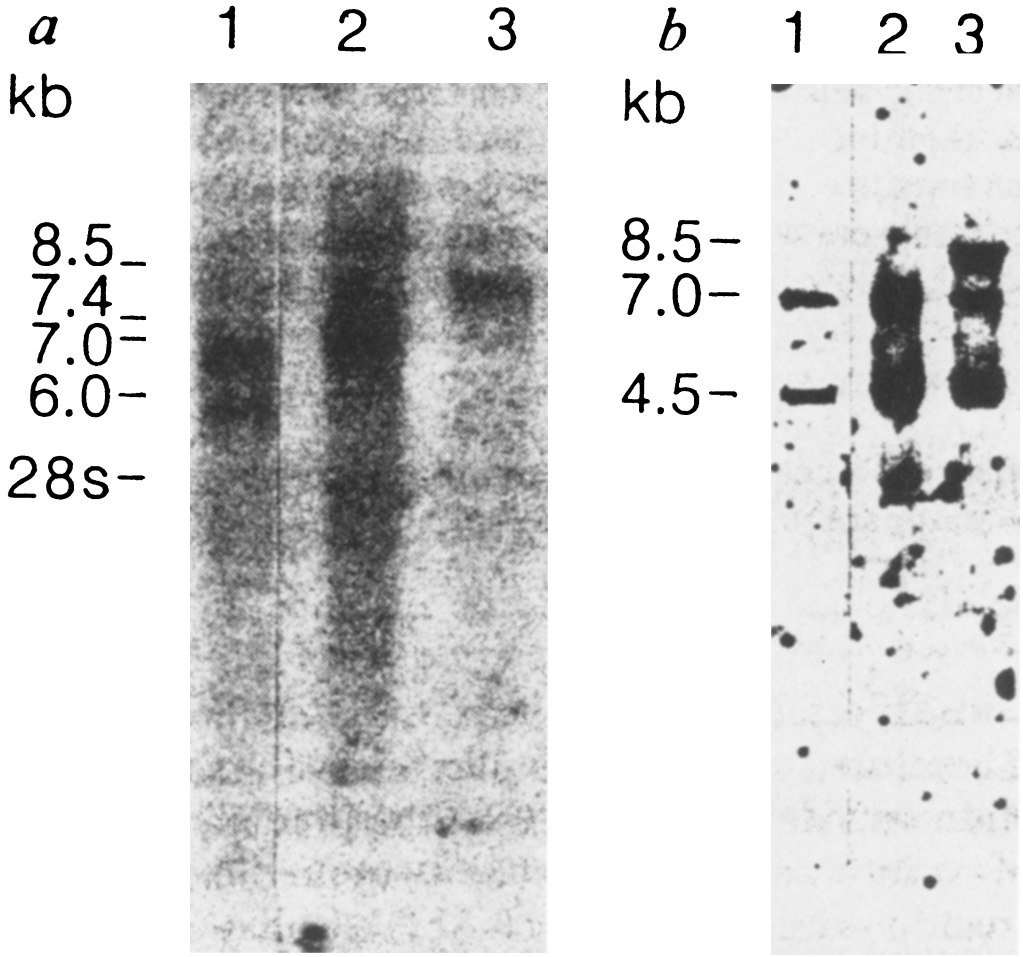

FIGURE 8. Northern blot analysis of mRNA from patient samples for the diagnosis of the *c-abl-bcr* translocation. The hybridization was performed with either a genomic *c-abl* probe (panel *a*) or a *bcr* cDNA probe (panel *b*). Lane 1, Philadelphia-chromosome-(Ph-) negative acute promyelocytic leukemia cell line HL-60; lane 2, Ph-positive acute lymphocytic leukemia (ALL) patient; lane 3, Ph-positive chronic myelogenous leukemia (CML) patient. Note that in panel *a*, lane 1, two mRNAs, transcribed from the normal *c-abl* gene, are shown with lengths of 6.0 and 7.0 kb. In lane 2, an aberrant 7.4-kb mRNA is shown, suggesting the presence of the translocation. In lane 3, an aberrant 8.5-kb mRNA is shown that is characteristic of the translocation seen in CML. In panel *b*, the normal *bcr* transcripts, 4.5 and 6.7 kb, are shown in lane 1. In lane 2 (ALL patient), only the normal bands appear, suggesting that the *bcr* sequences recognized by the probe are missing from the aberrant 7.4-kb *c-abl* message. In lane 3, the 8.5-kb mRNA is again detected, confirming that it contains both *c-abl* and *bcr* sequences. (From Kurzrock R, Shtalrid M, Romero P, et al. *Nature,* 1987; **325:** 631–35. With permission.)

B. Clinical Applications of the *ras* Oncogenes

The *ras* proto-oncogenes are activated by point mutations at codons 12, 13, or 61. From the three *ras* genes, the *K-ras* is mutated more frequently than the

FIGURE 9. PCR analysis of clinical samples for the diagnosis of the *c-abl-bcr* translocation. PCR was performed using the primer sets A, B, and probe C (left panel); primer sets A, B, and probe D (middle panel); and primer sets E, F, and probe G (right panel) (see also Figure 6). In all cases, the PCR product was Southern-blotted, hybridized with the radioactive probe, and autoradiographed. Molecular weight markers are shown on the left side of blots, in base pairs (bp). The loadings were as follows: lane designated K562 contains sample from a cell line that is positive for the Ph chromosome. Lane designated HL-60 contains sample from a cell line that is negative for the Ph chromosome. Lane 1 is a Ph-chromosome-negative patient. Lanes 2, 3, and 4 are Ph-chromosome-positive patients. Lane designated Sup B15 is an acute lymphocytic leukemia-Ph-chromosome-positive cell line. The blots are interpreted as follows. Left panel: a 200-bp PCR product is detected in the positive control and patient 3. This means that the abnormal mRNA is of the structure shown in Figure 6, upper panel. Middle panel: a 125-bp PCR product is obtained in patients 2 and 4. This means that the abnormal mRNA has the structure shown in Figure 6, middle panel. Right panel: a 307-bp PCR product is obtained in the control ALL cell line, indicating that the abnormal mRNA has the structure shown in Figure 6, lower panel. Note that no PCR product was produced in the Ph-chromosome-negative patient 1. (From Kawasaki ES, Clark SS, Coyne MY, et al. *Proc Natl Acad Sci USA* 1988; **85:** 5698–5702. With permission.)

H-ras and *N-ras*. The activated oncogene has reduced GTPase activity, a property that is thought to be linked to its tumorigenic potential.

Ras gene mutations are found with variable frequency in diverse malignancies.[37–41] Highest incidences are found in adenocarcinoma of the pancreas (90%), colon (50%), lung (30%), and in leukemias (30%). The diagnostic and prognostic value of *ras* mutations is currently unclear. In most cases, the presence of the mutation does not seem to correlate with the anatomical location, depth of invasion, level of differentiation, age, or sex of the patient. The notion that the presence of the mutation is an indicator of a more invasive tumor has not been confirmed in all cases but seems to hold true for lung adenocarcinoma.[42] The *ras* gene mutations have been studied extensively in colon carcinoma in efforts to develop models of the initiation and progression of the disease.[40]

Historically, *ras* gene mutations were identified with biological assays using DNA from tumors, which was used to transfect NIH/3T3 cells. Although very useful, these assays are not suitable for screening large numbers of samples because they are cumbersome and slow. Currently, PCR is used to amplify specific

segments of the *ras* genes that contain a mutation at codon 12 or 61. The PCR product is then used as a target in dot-blot hybridizations with a labeled probe that recognizes only the mutant PCR product (Figure 10).[43] For each mutation, specific hybridization probes have to be used to cover all possibilities. For example, the normal codon 12 of *K-ras* is GGT (glycine). Point mutations will result in the following possible combinations: GGA (gly), GGT (gly), GGC (gly), AGT (ser), TGT (cys), CGT (arg), GCT (ala), GAT (asp), and GTT (val).

Probes containing the mutations of the third nucleotide of the codon need not be used because the codons GGA, GGT, and GGC all code for glycine (degeneracy of genetic code). Thus, six probes are needed to cover all point mutations of the *K-ras* codon 12. Other simple methods for the detection of point mutations are described elsewhere. A newly developed technique based on exponential amplification, the ligase chain reaction, may also be applicable. The necessary probes for the study of *ras* point mutations are now commercially available. Mutant *ras* proteins can also be detected with monoclonal antibodies.[44,45]

Recently, it became possible to identify *ras* oncogene mutations in the stools of patients with colorectal tumors.[46] Cancer cells carrying the mutations are shed into the stool, where they can be identified by PCR amplification and Southern or dot-blot analysis. These findings can be used for diagnosis. This approach works whenever the tumor cells carry *ras* mutations, and this is true for 40 to 50% of patients. When the mutation is present, its correct identification in stool occurs with a frequency of about 90% (sensitivity). Of importance is that the test detects patients with adenomas that could be treated successfully at an early stage. The practical applications of this and similar approaches for other diseases (e.g., identification of oncogene mutations in urine of patients with bladder carcinoma or in the sputum of patients with lung carcinoma) remain to be seen.

C. Clinical Applications of the *myc* Oncogene

In Burkitt's lymphoma, virtually 100% of the patients have translocations that involve the *c-myc* proto-oncogene locus. In 80% of patients, the *c-myc* is translocated from chromosome 8 to 14, in an area that is in close proximity to the immunoglobulin heavy-chain locus. The other 20% of cases involve translocations that juxtapose the κ or λ light-chain genes with *c-myc* on chromosome 8.[12] The breakpoints in the *c-myc* locus are quite variable. The mechanism by which the translocations cause Burkitt's lymphoma is unknown. A close relationship seems to exist between tumorigenesis and partial deletions within regulatory elements of the first exon of *c-myc*. These deletions can now be studied by PCR, as shown in Figure 11. The deletions will cause the disappearance of the normal PCR product.

Genetic alterations of the *myc* oncogene have been found in many other malignancies. For example, in breast carcinomas the *c-myc* was found to be amplified 2- to 15-fold in 30% of patients.[47] Amplification and overexpression

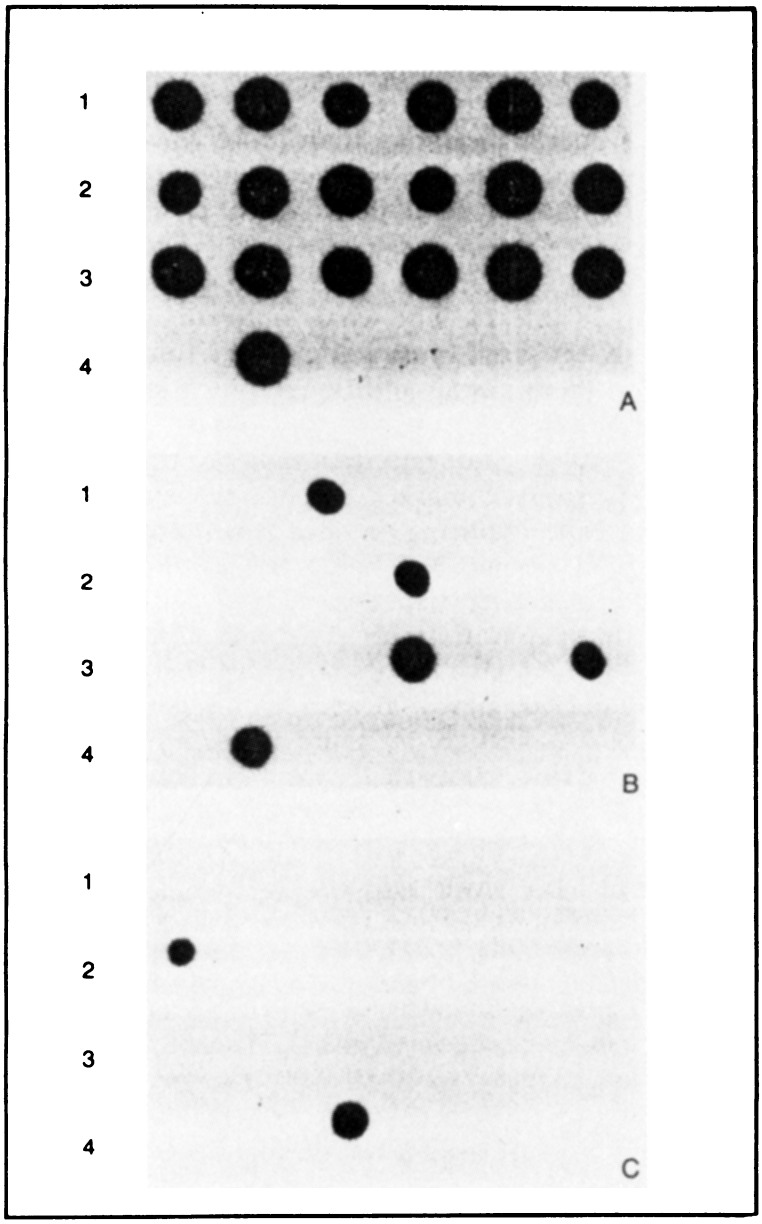

FIGURE 10. Dot-blot technique for the detection of point mutations in codon 12 of the *K-ras* oncogene in adenocarcinomas of the lung. DNA from 18 clinical samples was amplified by PCR and then dotted onto three different membranes, A, B, and C. Each membrane was then hybridized with a specific probe as follows. In A, the probe recognizes the wild-type codon-12, which is GGT (glycine); in B, the probe recognizes the mutant codon-12 TGT (cysteine); and in C, the probe recognizes the mutant codon-12 GTT (valine). In each membrane, there are four horizontal rows and six vertical columns. Row 4 contains only controls as follows. Column 1: no DNA added. Column 2: cell line NC1-H23 with a *gly* to *cys* mutation. Column 3: plasmid PCD-SW 11.1 with a *gly* to *val* mutation. Note the appropriate positive control spots in row 4 of each blot. The clinical samples gave the following results (rows 1, 2, 3). (A) All eighteen samples contained the wild-type allele of *K-ras*. (B) Four samples had the *gly-cys* mutation. (C) One sample had the *gly-val* mutation. (From Slebos RJC, Kibbelaar R, Dalesio O, et al. *N Engl J Med,* 1990; **323:** 561–65. With permission.)

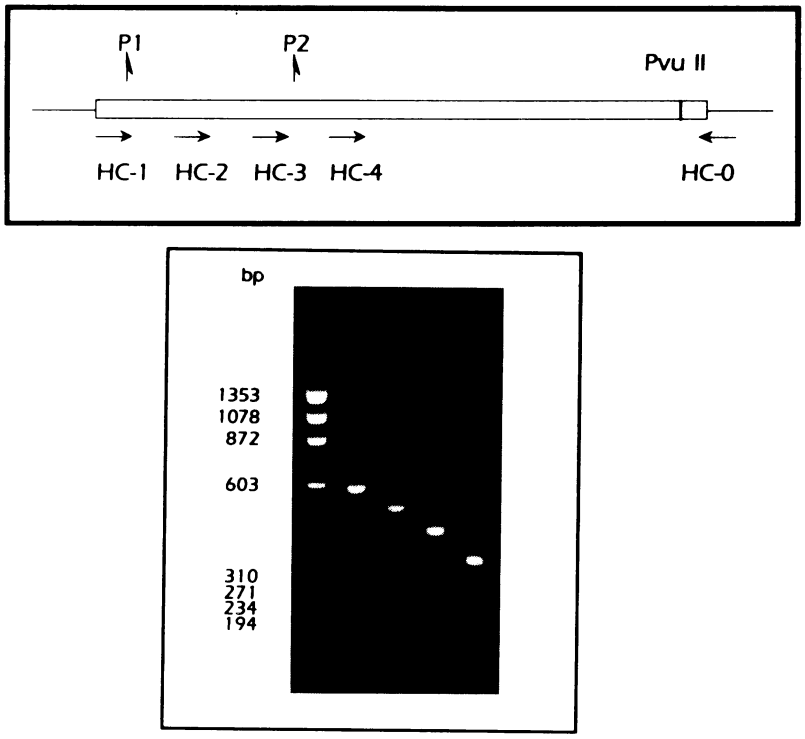

FIGURE 11. Upper panel: Schematic of human *c-myc* exon 1 indicating the two promoter regions (P1, P2). The five shown primers can be used in pairs (the right primer HC-0 in pair with one of the others) to generate PCR products of different sizes. These products can be detected by agarose gel electrophoresis (lower panel). Lane 1 has molecular weight markers. Deletions in the promoter regions are detected by the absence of PCR product. Figure was adapted from the Clontech catalog, and the primers are commercially available from Clontech Labs, Palo Alto, CA.

were also seen in multiple myeloma, colon, and lung carcinomas, and rhabdomyosarcomas (Figure 12 and Table 4).[9,48–50] *N-myc*, a gene homologous to *c-myc*, is amplified in tissues and cell lines from patients with neuroblastoma. About 40% of patients show amplification, sometimes up to 300-fold.[51]

It has been found that *myc* gene amplification is a bad prognostic indicator, and patients with amplification have shorter survival rates than patients with single-copy *myc* genes. Gene amplification is usually associated with increased mRNA and protein levels of the gene, but the protein is not mutated. Thus, the oncogenic consequences of amplification are due to overproduction of the normal protein molecule.[52]

D. Clinical Applications of the *c-erbB-2* Proto-oncogene

The *c-erbB-2* proto-oncogene, also known as *her-2* or *neu*, is a transmembrane protein that shows homology to the epidermal growth factor receptor (Figure 2).

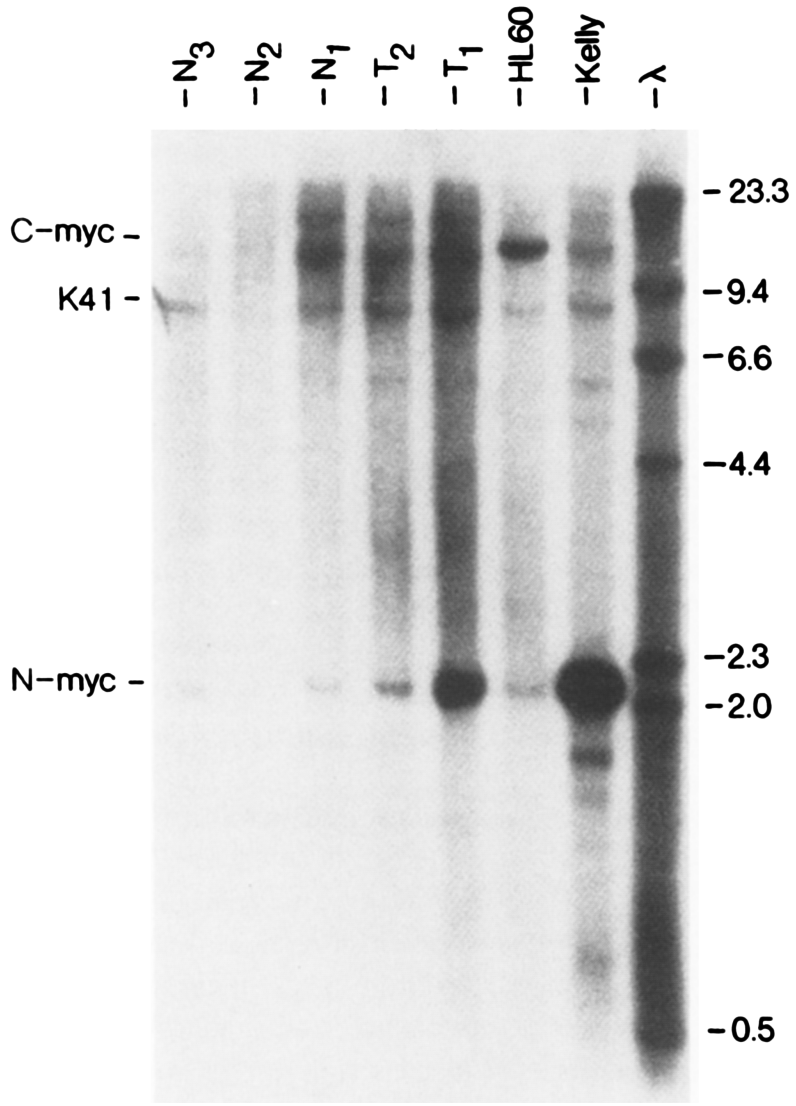

FIGURE 12. Detection of *myc* gene amplification by Southern blot analysis. DNA was digested with EcoRI; blotted; hybridized with a mixture of radioactive probes for *N-myc, c-myc*, and K-41 (control probe); then autoradiographed. Loadings were N_1, N_2, N_3 — three normal donors. T_2 and T_1 were embryonal and alveolar rhabdomyosarcoma, respectively. HL60 is a cell line with amplified *c-myc*. Kelly is a cell line with amplified *N-myc*, and λ are molecular weight markers in kilobars, as shown. Note the *N-myc* amplification (20-fold) in T_1. No amplification of *c-myc* is seen in either T_1 or T_2, and no amplification is seen in *N-myc* in T_2.

A number of studies have shown that this proto-oncogene is amplified in about 15 to 40% of primary breast and ovarian carcinomas.[53–56] The amplification results in elevated levels of mRNA and protein.

It has been suggested that amplification of this gene is associated with positive lymph node involvement and poor patient outcome (even in patients without nodal involvement)[57] and is thus a candidate predictor test for prognosis. Other studies failed to confirm this association.[54] However, there is reasonable agreement that

c-erbB-2 may be involved in the pathogenesis of the disease and may contribute to the development of distinct histologic types. The link between *c-erbB-2* and pathogenesis has prompted scientists to develop antibodies against the protein product that can be injected therapeutically to inactivate it.[24]

Recent studies have stressed the necessity of assessing *c-erbB-2* status at the DNA, mRNA, and protein levels.[53] This seems to be necessary because in some tumors increased mRNA and protein can be seen without the presence of DNA amplification. Amplification studies at the DNA and mRNA levels are performed with Southern or Northern blot techniques, respectively. Protein levels are assessed with Western blotting or immunohistochemistry. Examples are given in Figure 13. As mentioned in other cases of gene amplification, the amplified gene is found to have normal sequence, indicating that activation is due to overproduction of a normal protein molecule. Very recently, an ELISA assay for *c-erbB-2* protein has been developed and used to measure levels of the protein in tumors.[58]

E. Clinical Applications of the Retinoblastoma Gene

The tumor suppressor genes, one of which is related to retinoblastoma, *Rb*, are referred to as recessive genes because the presence of one normal allele is usually sufficient to prevent the expression of the malignant phenotype. Loss of function of the remaining normal allele, a process known as "loss of heterozygosity," has been observed in many tumor types, including retinoblastoma, osteosarcoma, Wilm's tumor, hepatoblastoma, and rhabdomyosarcoma.[6,59]

In hereditary retinoblastomas and osteosarcomas, the patients are heterozygous for the *Rb* locus at the germline level.[60] One somatic mutational event is needed to produce homozygosity. However, in sporadic retinoblastoma and in all other tumors in which the *Rb* locus is found to be homozygously altered, the *Rb* inactivation at both loci is due to purely somatic events. This holds true for tumors like small-cell lung carcinoma and bladder and breast carcinomas.[61]

Rb gene inactivation can occur at one allele as a result of loss of a chromosome, a chromosome arm, or a small band. Some of these changes may be cytogenetically visible. The loss of the other allele can occur through point mutation, insertion, translocation, or deletion.

Although alterations of the *Rb* gene have been observed in many tumor cell lines, eg., small-cell lung, bladder, breast carcinomas, and in leukemias, studies on actual tumors are limited. In one study involving 77 breast carcinoma tumors, about 19% had structural abnormalities of the gene.[62] The gene product, a 110-kDa nuclear phosphoprotein, was found to be absent from the cells having the gene structural abnormalities. Although the structural changes observed in the *Rb* gene have been implicated in the etiology of tumor development, no prognostic value has been proposed for these findings. Simple tests for the detection of heterozygosity could aid in the identification of individuals who are predisposed to the development of retinoblastoma and other malignancies.

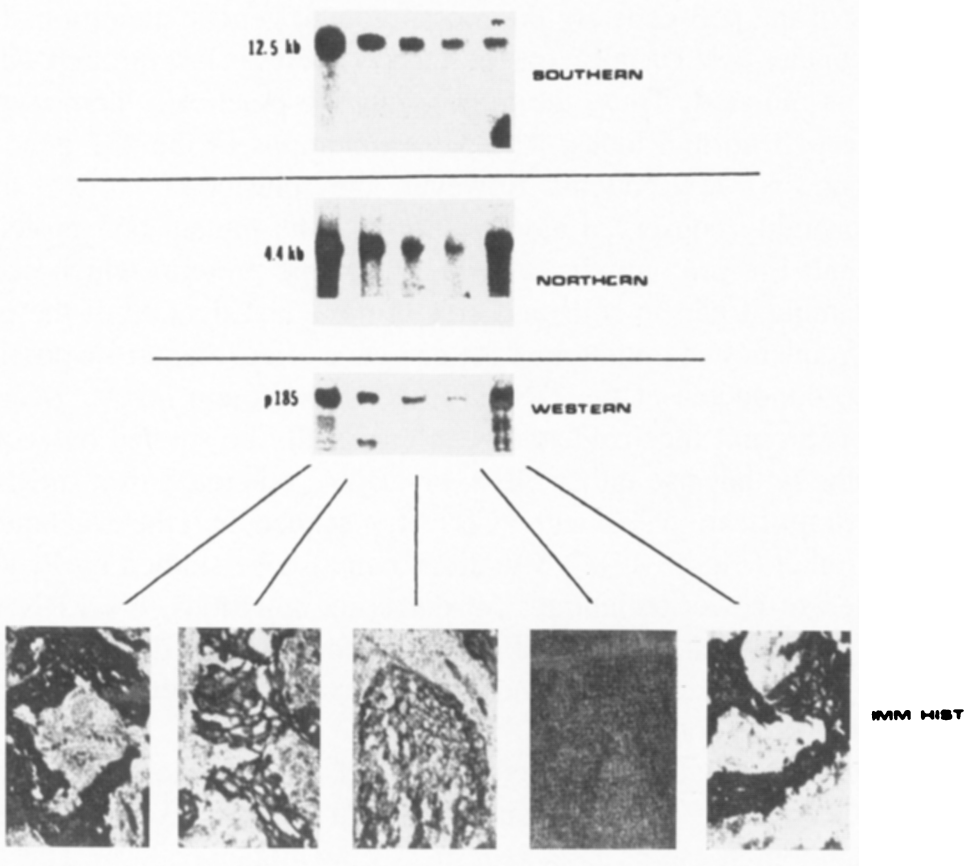

FIGURE 13. Study of Her-2/*neu* gene amplification and expression. Tumor DNA was analyzed by Southern or Northern blots. Protein levels were analyzed by Western blots or immunohistochemistry. Southern blot: the various samples had *neu* copy numbers of 20, 5-20, 2-5, 1, and 1, respectively, from left to right. Northern blot: the various bands had absorbance readings of 4.3, 1.4, 0.9, 0.2, and 2.8, respectively, from left to right. Western blot: The various bands had absorbance readings of 2.2, 1.1, 0.6, 0.12, and 2.1, respectively, from left to right. Immunohistochemistry: the intensity of staining correlates closely with the Western blot data. These data show that for the first four samples, there is correlation between DNA amplification, mRNA, protein levels, and immunohistochemical staining. However, the fifth sample with a normal DNA copy number had very high levels of mRNA and protein and showed intense immunohistochemical staining. (From Slamon DJ, Godolphin W, Jones LA, et al. *Science* 1989; **244:** 707–12. With permission.)

F. Clinical Applications of the p53 Gene

The p53 gene is located on the short arm of chromosome 17 and encodes a 53-kDa nuclear phosphoprotein (p53). This protein binds viral oncoproteins, including the SV40 large T antigen, with very high affinity. It is speculated that the wild-type p53 binds to a cellular homolog of SV40 large T antigen as part of its role in the control of the cell cycle. The mutant p53 loses its binding ability, an event that is thought to be critical in deregulating the cell cycle and thus initiating the malignant transformation.

Mutations of the p53 gene are the most frequent genetic alterations found in human malignancies.[63,64] Usually, one of the p53 alleles is lost through a deletion, and the other is mutated. Thus, the tumor cells are practically homozygous for the loss of the p53 normal locus. The point mutations of the p53 gene are not localized in one or a few codons. However, they cluster in exons 5 to 9, the exons that are highly conserved among species. The mutant p53 protein has a much longer half-life in comparison to the wild-type protein, which causes accumulation of mutant p53 in cells and aids in the quantification of the protein.

The high frequency of mutation of the p53 gene offers diagnostic possibilities. Studies can be conducted at the DNA, mRNA, or protein levels. Because the point mutations are not localized, they can classically be studied by sequencing the gene. Methods that use either DNA or cDNA generated from mRNA start with an initial amplification step with PCR. After sequencing, the exact mutational event can be found (Figure 14).[65] Mutations can also be studied by PCR amplification coupled to newer techniques for detecting mutations, e.g., RNAse protection assay, single-strand conformational polymorphism, or denaturing gel electrophoresis, with the hydroxylamine-osmium tetroxide method or with constant denaturant gel electrophoresis.[66]

The p53 protein can also be studied with panels of monoclonal and polyclonal antibodies that recognize wild-type and/or mutant p53.[67–70] We and others have developed immunometric-type assays for measuring mutant p53.[71,72] These assays are very effective in quantifying mutated p53 in tumor cell line lysates and tumor tissue homogenates. In a recent study involving 212 human malignant tumors, 76% had accumulated mutant p53 as demonstrated by immunohistochemistry.[73]

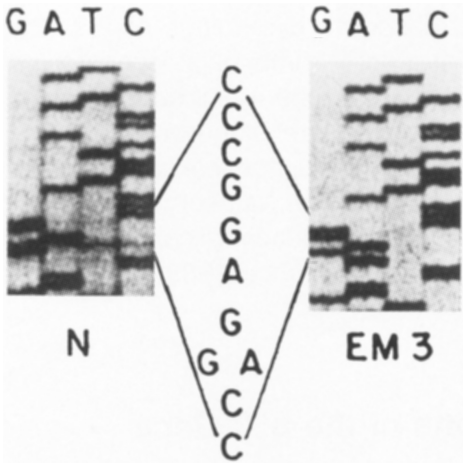

FIGURE 14. Sequencing data for a segment of the p53 gene. Note the normal sequence, CCG, in the left panel and the point mutation that results in a G to A transition (right panel). Such mutations can inactivate the p53 tumor suppressor gene. (From Feinstein E, Cimino C, Gal RP, et al. *Proc Natl Acad Sci USA* 1991; **88:** 6293–97. With permission.)

Tumor types were breast, colon, stomach, bladder, and testicular, as well as sarcomas and melanomas. The pattern of p53 expression in breast cancers, as judged by immunohistochemistry, can be reportedly used for tumor subclassification.

The presence of mutant p53 protein in such a high percentage of diverse tumors[73,74] led us to speculate that p53 may be a tumor marker if it appears in serum. This possibility is currently under investigation. Alternatively, proposals have been made that mutant p53 is immunogenic for the host, which would mean that the host would develop antibodies against mutant p53 that could be measurable in serum. Such antibodies were found to exist in about 9% of patients with breast carcinoma.[75] More extensive studies are now underway to address the possible presence of p53 antibodies in other malignancies. If present, these antibodies may be useful for diagnosis and monitoring.

Recently, it has been reported that p53 gene mutations can be detected in tumor cells found in urine sediment from patients with bladder carcinoma.[76] This finding could, in theory, be used as a diagnostic tool and as an aid in monitoring patients who have undergone surgery, for early detection of relapse. The practical implications of these findings remain to be seen.

The most encouraging results from recent clinical trials suggested that p53 mutant protein was a good predictor of prognosis in patients with breast and prostate cancer.[77,78] In both types, accumulated mutant protein was an unfavorable sign, leading to shortened survival rates. It seems that p53 is on the verge of being used routinely as a prognostic test for these cancers.

IX. CONCLUSIONS

Cancer is a leading cause of death, and research in this field is intensive. There was always the view that tumor markers, specific substances that are released by the tumor into the circulation, must exist and that their identification could open the way for cancer diagnosis, monitoring, treatment, and prognosis. The tumor markers that have been identified during the last 20 to 25 years suffer from lack of specificity and are not suitable for screening or diagnosis.[79] These tumor markers are either oncofetal antigens (normal constituents of the fetus) or physiological substances that are present in biological fluids in the normal state but at low concentrations. Abnormal control of gene expression in cancer cells can cause their reappearance in serum (for oncofetal antigens) or their increased, inappropriate production. However, these biochemical changes are now considered late epiphenomena and are not involved in the pathogenesis of cancer.

Oncogenes and tumor suppressor genes or their products could be specific tumor markers for the following reasons:

1. The alterations in many of these genes are considered pathogenic and must be present at the initiation stage of cancer.

2. Alterations in some genes are found only in specific cancer types, whereas in other genes they are found in many types, offering possibilities for both tumor-specific and non-tumor-specific diagnosis.
3. In some instances, these genetic alterations produce unique products (mRNA, protein) that do not exist in the normal state and are thus truly specific indicators of malignancy.

As might be expected, these markers have their difficulties as well. Currently, with some exceptions mentioned,[46,76] it is not clear whether the abnormal genes or gene products are present in the circulation, urine, sputum, or feces. If this does not happen, the usefulness of the marker will be restricted to studies on tumor tissue for subclassification and prognosis, but not for diagnosis. With the exception of rare hereditary forms of cancer, the genetic alterations in cancer are somatic, which means that they are found only in tumor cells but not in normal cells, again necessitating tumor sampling for study.

Additionally, specific genetic abnormalities often exist only in a percentage of tumors. For good sensitivity we need to use panels rather than individual tests. The best genetic markers are those present in malignant or even in premalignant cells at a very early stage. Studying such cells will ensure that the tumor is diagnosed while there are good prospects for successful therapy.

Some of the genetic alterations identified in oncogenes and tumor suppressor genes are already in use for tumor subclassification and prognosis. With the exception of chromosomal translocations, these genetic markers are not specific and have relatively low sensitivity. But this is only the beginning. We can envision with optimism that in the future, with use of already existing powerful detection and amplification techniques, we will be able to detect minute amounts of tumor-specific genetic markers (DNA, mRNA) or their protein products in the circulation. These markers could be released during cell turnover and be used for diagnosis. While researchers from other branches of sciences are now trying to identify new and more useful genetic alterations in cancer, the clinical chemist should be ready to transfer those advances from the bench to the bedside.

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