COLLECTIVE REVIEW

THE EMERGING GENETICS OF CANCER

Michael A. Skinner, M.D., *and* J. Dirk Iglehart, M.D., *Durham, North Carolina*

WITH THE EXPLOSIVE PROGRESS made in the field of molecular biology during the past decade, researchers have been successful in dissecting some of the mechanisms by which the growth of cells is regulated. In particular, much attention is being directed to discovering the fundamental genetic events that underlie the development of neoplastic growth. A body of evidence supports the assertion that the basic lesion of the malignant cell is an abnormality in the genome, which results in unregulated growth of cells. This notion is supported by the hereditary predisposition to certain types of carcinoma (1), the recognition that chromosomal aberrations are commonly found in malignant cells (2) and the observation that known mutagenic chemical agents also tend to be carcinogenic (3, 4). Evidence is accumulating that a particular class of genes, the oncogenes, are especially important in the initiation and growth of malignant lesions in humans.

Oncogenes exist normally in every eukaryotic species that has been investigated (5–8). The usual function of oncogenes is to regulate cellular growth or embryonic development (9, 10), but if they are inappropriately expressed, carcinoma may result. More than 40 of such genes have been discovered, and, in several types of carcinoma, the amplification of particular oncogenes has been associated with disease that has a worse prognosis (11). It is likely that increased expression of oncogenes will become a clinically useful indicator of biologic activity and aggressiveness in some systems of tumors. In this review, the history, mechanisms of action and clinical aspects of oncogenes will be discussed. Because much of what is known about the way in which oncogenes induce carcinoma has been learned from the study of tumors with familial variants, such as adenocarcinoma of the colon, emphasis is placed on these systems.

From the Department of General and Thoracic Surgery, Duke University Medical Center, Durham, North Carolina.

Reprint requests: Dr. Michael A. Skinner, Department of Surgery, Box 3873, Duke University Medical Center, Durham, North Carolina 27710.

HISTORY AND MECHANISMS OF ACTION

The study of the molecular basis of neoplasia was initiated in 1911 with the seminal work of Peyton Rous (12). He demonstrated that avian sarcomas could be transmitted from one bird to another by injecting a cell-free, filtered extract of the tumor. The responsible agent is the Rous sarcoma virus, which is a member of the retrovirus family. These viruses are unique in that the genetic information is carried on strands of ribonucleic acid (RNA) rather than deoxyribonucleic acid (DNA). For the virus to replicate, a DNA replica of the RNA template is first constructed by the enzyme reverse transcriptase. The double stranded molecule of DNA is then integrated into the genome of the host and transcribed to manufacture the viral proteins necessary for replication. Acutely transforming RNA viruses similar to the Rous sarcoma virus have been subsequently found in many eukaryotic species (7, 8, 13). The oncogenic activity has been traced to the presence of a single gene, the oncogene, whose function is completely unassociated with the ability of the virus to infect and replicate within the host (14).

The existence of the viral genes allowed the early production of genetic probes, which were initially used to investigate the possible existence of other virally induced tumors. Investigators were surprised when experiments with nucleic acid hybridization demonstrated the presence of genes in uninfected, normal, mammalian cells, which are homologous to the viral oncogenes (15, 16). These normal genes are called proto-oncogenes or cellular oncogenes, and they are highly conserved through phylogeny. It is probable that, in the past, they were acquired by the

viruses through genetic recombination (17). With the improvement of molecular techniques, many additional cellular genes that exhibit less homologic characteristics with the known viral oncogenes have been found.

One productive method for discovering new transforming genes is the NIH-3T3 focus-forming assay. The 3T3 cell line is an immortalized line of murine fibroblasts, which, unlike normal cells, will not enter the senescent phase after a number of generations. The cells are grown in a monolayer and the DNA of interest is transfected into them in the presence of calcium phosphate. The cells normally stop dividing when they become confluent on the plate, but if a transforming gene is absorbed and expressed by a cell, it will lose this normal cell surface contact inhibition. The cells will then begin to pile and form a visible focus of transformation. The cells may be grown and the particular gene of interest can be cloned and identified. The first human lesion to be analyzed in this manner was the T-24 cell line of carcinoma of the bladder. Here, the responsible gene was homologous to the transforming sequence contained within a murine acute transforming virus, the Harvey sarcoma virus (18). This virus was first discovered by Jennifer Harvey, and the viral oncogene that it contains is named Harvey-*ras* (Ha-*ras*). Molecular dissection of the T-24 transforming gene has revealed that the gene differs from the normal human cellular Ha-*ras* gene by a single point mutation in the 12th codon (19, 20). This results in a significant alteration in the secondary structure of the transcribed protein, which causes unregulated growth of the cell (21, 22). A number of other mutated oncogenes have been found in association with human tumors using the NIH-3T3 focus assay (23). In general, the genes are members of the *ras* family of oncogenes and include the Ha-*ras*, the Kirsten *ras* gene (Ki-*ras*) and the N-*ras* gene.

A proto-oncogene does not necessarily need to be mutated for its expression to result in transformation. Simple overexpression of the normal, unmutated gene can cause a deregulation of cellular mitosis. For example, if the normal protein product from the Ha-ras proto-oncogene is microinjected into 3T3 fibroblasts, the cells will assume the neoplastic phenotype (24). In addition, if the normal gene is linked to a very strong viral promoter element and transfected into the fibroblasts, then foci of transformation will result (25). This is the case with most of the known proto-oncogenes, demonstrating that normal cellular oncogenes may cause transformation simply by being inappropriately overexpressed.

The increased expression of an oncogene may be associated with an increase in the number of copies of the gene within the cell. This phenomenon is known as gene amplification. The first oncogene found to demonstrate significantly aberrant expression in a human solid tumor system was c-*myc*. This gene was initially discovered to be amplified 20 to 76-fold in a series of cell lines of small cell lung carcinoma (SCLC) (26). In three of the five lines, karyotypic analysis demonstrated the presence of double minute chromosomes and homogeneously staining regions, which are known cytologic markers of gene amplification (27). Analysis of messenger RNA (mRNA) indicated an increased expression of the gene, and all of the cell lines were of a variant class of SCLC that is less differentiated and more malignant than its normal SCLC counterparts (28).

Another mechanism by which proto-oncogenes may elicit carcinoma is by the phenomenon of genetic rearrangement and insertional mutagenesis. Here, gene expression is increased by the inappropriate placement of a highly active promoter element in front of the gene. This phenomenon was initially observed in carcinomas caused by viruses that do not possess a transforming oncogene. Rather, carcinoma is induced when the strong viral promoter element is placed within the genome of the host, so that constitutive activation of a normal cellular oncogene occurs (5). It remains controversial whether or not this process occurs in human malignant invasions. In Burkitt's lymphoma, however, a chomosomal translocation is routinely found to involve the eighth chromosome near the locus of another proto-oncogene, c-*myc*. This human gene is homologous to the transforming oncogene found in the avian myelocytomatosis virus and has been found to exhibit increased expression in a number of human tumors. In cells of Burkitt's lymphoma, the gene is always juxtaposed downstream from a highly active immunoglobulin promoter (29–31). This position results in markedly increased expression of c-*myc*.

The results of a recent in vitro experiment support the assertion that the increased c-*myc* expression is causally related to this disease. Burkitt's lymphoma is a B cell neoplasm that exists in several epidemiologically distinct forms. One of the forms, endemic Burkitt's lymphoma found in Central Africa and New Guinea, is associated with the Epstein-Barr virus. When

normal human B lymphoblasts are infected with the virus, they become immortalized but will not normally grow to form tumors in nude mice. If, however, the c-*myc* gene is subsequently placed into the immortalized cells, the lymphoblasts acquire the ability to form such tumors. In addition, the cells exhibit other markers of biologic aggressiveness, such as the ability to grow in vitro with reduced serum (32). Thus, it is likely that the increased expression of the c-*myc* gene in some way enhances the aggressivenss of Burkitt's lymphomas.

Oncogenes exert mutagenic effects onto cells by coding for proteins that act to uncouple the cell from its normal regulatory signals. The oncogene-coded proteins thus far characterized are all members of relatively few functional groups and act within a complicated circuit that serves to transduce an extracellular message into the nucleus to govern cell division. The circuit may be initiated by the interaction between a growth factor and the extracytoplasmic component of its receptor, resulting in an allosteric alteration through which the intracellular component of the receptor activates poorly characterized second and third messengers, often through the activity of tyrosine kinase. The messengers ultimately interact with intranuclear proteins that, in some way, initiate mitosis and cell division. Any of the proteins in the circuit may be encoded by oncogenes, and, if altered, a growth signal may be sensed when it is not actually present.

A number of these proteins are homologous to known growth factor receptors or portions of the receptors. For example, the viral *erb*-b portion of an oncogene is a truncated form of the epidermal growth factor (EGF) receptor (33), lacking the ligand binding portion of the extracytoplasmic component (34, 35). The intracellular portion of this oncogenic protein is continually in the allosteric configuration taken by the intracellular portion of the normal EGF receptor after it has bound to the EGF. This results in constitutive activity of tyrosine kinase and, presumably, increased second message activity. Thus, the cell senses a signal to replicate in the absence of the growth factor. Similarly, the recently described c-*erb*-b2 oncogene product is a receptor that is homologous to the EGF receptor (36, 37). The ligand is as yet unknown, but increased expression of the gene has been described in a number of cell lines of human carcinoma (38, 39), and gene amplification has been reported to be associated with more aggressive carcinomas of the mammary gland (40). The increased number of receptors may produce an enhanced second message activity in the presence of low levels of growth factor. This condition would result in unregulated growth.

Another mechanism by which the activation of oncogenes may interfere with the normal cellular response to growth factors is the encoding for a protein by a gene that is itself a growth factor or an analog. The c-*sis* proto-oncogene, for example, codes for platelet derived growth factor (PDGF) (41). Furthermore, a number of cell lines of small cell carcinoma secrete bombesin, to which the cells also possess receptors (42, 43). These cells exhibit increased growth in the presence of this peptide, and this response is abrogated in the presence of antibombesin antibodies (44). Thus, the hormone acts on the cells of the tumor in an autocrine manner with a positive feedback loop (45). A similar phenomenon is present in the MCF-7 cell line of carcinoma of the breast, in which insulinlike growth factor-1 is an autocrine factor (46).

One ubiquitous second messenger system that has recently been the subject of much research is the polyphosphoinositide system (47). These lipids are located in the cellular membrane and are known to be intermediaries that interact with a variety of hormones, neurotransmitters and growth factors. The molecular events that occur in these interactions are now known, and probably involve known products of oncogenes. The c-*myc* and c-*fos* oncogenes, for example, exhibit increased expression when the system is activated (48). Initially, the binding of a cell surface receptor to its ligand causes the activation of the enzyme phospholipase C. This activation involves an intermediary protein from the family of G proteins and results in the production of phosphoinositides, which are known second messenger molecules. The G proteins are so called because they exhibit guanosine triphosphate binding activity as well as guanosine triphosphatase activity.

The *ras* proto-oncogene product possesses G protein activity, and evidence shows that the activated *ras* oncogene transforms the cell by short circuiting the second messenger system. First, the cellular production of the phosphoinositides by phospholipase C in response to the administration of growth factors is enhanced when the *ras* gene is introduced into the cell (48). Furthermore, when the acutely transforming mutated *ras* oncogene is placed within a cell, the production of the second messenger phosphoinositide molecules increases markedly, even in the absence of exo-

genous growth factors (49). Thus, it is likely that the activated *ras* oncogene that is found in a number of human tumors acts in a constitutive manner, continually stimulating phospholipase C to produce second messenger molecules to initiate mitosis. The cells containing this gene behave as if undergoing stimulation by growth factors when they are not.

The predominant theory of neoplastic progression is that it is a gradual phenomenon in which cells become more dysplastic with time. This process continues until frank malignant invasion occurs (50). On a molecular basis, evidence shows that two or three separate genetic events must occur before a cell becomes frankly malignant (51, 52). Also, the propensity of a cell to metastasize is different from the ability to form tumors. This concept is supported by the well established fact that a tumor has many different cell populations that exhibit different phenotypic characteristics, such as growth rate and metastatic potential (53, 54). The relationship between these biologic factors and cellular oncogene expression is not known, but it is likely that a population of cells exists upon which a growth advantage is conferred by the expression of the aberrant genes. The cells may grow preferentially and metastasize as the disease progresses.

Clinical examples show that the expression of known oncogenes appears to enhance the aggressiveness of malignant cells, rather than simply cause their transformation. An activated Kirsten c-*ras* gene, for example, has been recently found in a metastatic variant but not in the primary cell line from a murine T cell lymphoma, suggesting that the gene has conferred the metastatic potential on this previously transformed cell line (55). In addition, it has been reported recently that the transfection of the *ras* gene into previously transformed cells results in a more invasive phenotype (56). Another group has reported finding a significantly higher expression of the *ras* protein product in carcinomas of the breast having significant axillary metastatic disease than in tumors with less extensive disease at the time of resection (57). Here, the same histologic phenotype was found in both the primary and metastatic tissues, and none of the *ras* protein was found in the normal tissues. The results from several other studies of cell lines of tumors and resected specimens have failed to show a relationship between the expression of *ras* oncogenes and metastatic capability (58–60), but this may be related to the inability of this particular oncogene to confer metastatic potential onto certain cell lines (61). In these tumors, it is likely that some other regulatory genes are enhanced or promoted to cause metastasis. Indeed, it has been recently reported that the expression of c-*fos* was markedly increased in the clones of cells of carcinoma of the breast that exhibited metastatic potential in rats (62). It is the potential of oncogenes to affect the biologic aggressiveness of cells of tumors that is spurring the development of methods to assay oncogenic activity in the clinical setting.

CLINICAL ASPECTS

Neuroblastoma is the most common solid tumor in children, composing 25 to 50 per cent of all tumors in children less than 30 months of age (63). The disease is believed to arise in the neural crest, and the most common site of origin is the abdomen, in which the tumor arises either from the adrenal gland (40 per cent) or from the paraspinal ganglion (25 per cent). Approximately 25 per cent of patients with neuroblastomas are diagnosed within the first year of life, and 50 to 60 per cent are found in patients more than two years of age. A total of 60 to 70 per cent of all patients with neuroblastoma will have evidence of metastatic disease at the time of initial diagnosis. This is a clinically important disease, which has been unresponsive to the standard multimodality therapies used successfully to treat several other childhood malignant lesions.

Recently, however, some progress has been made in dissecting the molecular events that determine the biologic aggressiveness of neuroblastoma. First, it was discovered that human cell lines of neuroblastoma have increased numbers of copies of a gene that is homologous to c-*myc*. The gene is known as N-*myc*. Subsequent analysis of surgically resected neuroblastomas has revealed that N-*myc* is also amplified in the primary tumor, indicating that this is not a phenomenon resulting from in vitro cell culture. Indeed, it has long been known that neuroblastomas possess the double minute chromosomal elements and homogeneously staining regions characteristic of genetic amplification (64, 65).

A series of 89 patients with untreated neuroblastomas was reported (11). Analysis of the resected specimens revealed that amplification of the N-*myc* gene was found in 34 (38 per cent) of the tumors, with the number of copies varying from two to 300. A significant association was found between the multiplicity of genes and higher stage of disease at the time of diagnosis (66). In particular, amplification was detected in two of 16 tumors in Stage II, 13 of 20 in Stage III

and 19 of 40 in Stage IV. The 13 tumors of Stages I and IV-B all exhibited unamplified, single copies of the gene. The results of clinical follow-up studies of the patients indicate that the likelihood of being a disease-free survivor 18 months after diagnosis with N-*myc* copies of one, three to ten and greater than ten was 38, 16 and zero per cent, respectively. Thus, although the patients in this study were all treated with the appropriate therapy for the stage of disease, the number of N-*myc* copies was significantly correlated in an inverse manner with the disease-free survival period.

In addition, the degree of gene amplification also correlated with the disease-free interval within a given stage of disease. For example, the two patients with Stage II disease whose tumors demonstrated an increased number of copies of the N-*myc* gene had recurrences with widespread metastasis within seven months, while only one of the remaining 14 patients with this stage of disease had a recurrence.

In another study, it was verified that actual N-*myc* expression was increased in concert with the amplification of the number of copies of genes in a majority of high stage neuroblastomas. The verification was done by measuring amounts of mRNA in the tissue, and in one instance, increased expression was found in the absence of gene amplification (67). Additionally, a marked heterogeneity among the cells of the tumor was found, regarding the amplification and expression of N-*myc*, and these characteristics were present in cells that were morphologically more poorly differentiated. Another report demonstrated that the expression of N-*myc* is increased in cells cultured from a recurrent tumor after treatment, when compared with the cells cultured from the initial, untreated tumor, suggesting that the increased N-*myc* expression confers an advantage on the cells, making them resistant to treatment (68). Whether the increased *myc* expression is a cause or an effect of increased aggressiveness of tumor is not known. Indeed, the size of the amplified chromosomal segment ranges in size from $2(10)^5$ to $2(10)^6$ base pairs. This segment may contain many genes, and N-*myc* may simply be a marker for a clone of cells that possess a growth advantage because of the presence of some other gene in the amplified segment.

An animal tumor system supports the assertion that solitary increases in *myc* expression result in a more malignant tumor. Neuroblastomas are reliably produced in neonatal rats by the transplacental instillation of the chemical carcinogen ethylnitrosurea. Analysis of the carcinogen induced tumors of rats uncovered a unique transforming gene, *neu*, named after the neuroblastoma in which it was discovered (69, 70). This gene is probably the rat homolog of the human c-*erb*-b2 gene and scores positive on the NIH-3T3 focus-forming assay. The subsequent introduction of the human N-*myc* gene into the cells of neuroblastomas in rats transformed by *neu* confers on them an increased growth rate as well as the ability to metastasize (71). Interestingly, there is also a concomitant decrease in the expression of the Class I major histocompatibility complex antigens. It is provocative to postulate that the increased production of the *myc* protein somehow reduces the ability of the neoplastic cells to elicit an immune response, allowing them to grow more aggressively.

The specific mechanism by which the *myc* protein affects biologic behavior is unclear. It is known that the protein resides in the nucleus and that the level of protein within 3T3 fibroblasts is increased more than fortyfold by stimulation with platelet derived growth factor (72). Also, in both normal and transformed cells, its expression is stimulated by activation of the phosphoinositide second messenger system (48). Finally, independent stimulation of the *myc* production partially abrogates the reliance on PDGF for growth of cells. Thus, the *myc* protein exhibits increased activity after initiating growth and second messenger signals. The *myc* protein may be the putative third messenger that interacts with the genome of the host to initiate cell division after a mitogenic stimulus.

The study of the embryonal tumors, retinoblastoma and Wilms' tumor, has elicited evidence of another clinically important molecular mechanism of carcinogenesis. Each tumor may present in either a familial, bilateral or sporadic manner. In general, both diseases will occur much earlier in the kindreds than in the population at large. Statistical analysis of this phenomenon, as well as the difference in the incidence of disease between the familial and sporadic groups, supports the assertion that Wilms' tumor and retinoblastomas result from two random, independent genetic events (72, 73).

Wilms' tumor accounts for 10 per cent of all pediatric neoplasms and is an example of a neoplastic disease that is responsive to modern therapeutic methods. Although early in this century less than 10 per cent of children afflicted with the disease were cured, today 90 per cent of these pa-

tients may be expected to recover (74). The disease generally presents in a sporadic manner, but kindreds exist in whom the disease is inherited in an autosomal, dominant pattern with reduced penetrance. In addition, the disease is associated with many congenital abnormalities, principally involving the genitourinary tract. An association with aniridia has been found in approximately one in 43 patients with Wilms' tumor, and the results of study of the patients have provided valuable clues regarding the molecular mechanisms of the disease. In particular, the genetic material from the short end of chromosome 11 is deleted from the gene line of these patients (75, 76). Subsequently, karyotypic analysis of other Wilms' tumors not associated with aniridia have shown a similar deletion (77, 78). In these patients, however, the deletion exists only in the cells of the tumor and not in the somatic tissues. Analysis with sensitive gene probing techniques has demonstrated the absence of genetic material from 11p in some tumors that do not show the deletion on routine karyotypic analysis (79, 80).

Thus, the absence of genetic material on the 11th chromosome associated with Wilms' tumors is common. This phenomenon is likely to be one of two genetic events necessary for tumorigenesis and may represent the loss of a carcinoma suppression gene, which allows the progression of the disease. Indeed, it has been shown recently that the introduction of a normal 11th chromosome segment into cultured cells of Wilms' tumor completely suppresses their ability to form tumors in nude mice (81). No differences were observed in markers of transformation, such as characteristics of cellular structure or the distribution of fibronectin on the surface of cells. The added genes simply decrease the biologic aggressiveness.

Similarly, retinoblastoma represents only 1 per cent of pediatric solid tumors, but it has provided an important model for understanding the genetics of carcinoma. The familial variety of the disease is observed in 40 per cent of patients and is an autosomal dominant trait with high penetrance. Most of the patients will have bilateral involvement, while only one-third with bilateral disease will have a family history. In the latter patients, it is probable that new germinal mutations have occurred (82). Those who have familial retinoblastomas have a loss of genetic material from chromosome 13 in all of their somatic tissues, while only the tumor lacks this material in patients afflicted with the sporadic form of the disease (83, 84). This loss is probably the first of the two genetic events necessary for transformation (81). Because each event occurs randomly and is distributed in a Poisson manner, as in Wilms' tumor, the patients who are born with the first event have a markedly increased probability that both events will eventually occur. Indeed, it is very likely that at least one retinoblast will experience the second step, which is probably the loss of the other allele of chromosome 13, with the resultant production of a retinoblastoma. A gene has been recently cloned from the chromosome region 13q14, which is normally expressed in retinal cells but is either aberrantly expressed or unexpressed in cells of retinoblastoma (85, 86). This may be the putative retinoblastoma susceptibility gene, whose absence results in formation of tumor. Experiments are under way to determine if introduction of the gene into the transformed cells reverses the neoplastic phenotype or otherwise changes their biologic characteristics.

A similar picture is developing in the case of carcinoma of the colon. Adenocarcinoma of the colon is the second most common malignant condition in the United States. Although the vast majority of the tumors occur sporadically, there is a genetic variant. Familial polyposis coli is an autosomal, dominant, inherited syndrome in which susceptible individuals develop adenomatous polyps of the colon (87). The process generally begins in adolescence, and the patient ultimately develops thousands of polyps in the large intestine. The polyps are precancerous lesions, and if untreated, there is virtually a 100 per cent likelihood that carcinoma will ultimately develop.

Sensitive genetic analysis of an affected kindred reveals that the somatic tissues of patients with familial polyposis coli possess an interstitial deletion of a segment in the fifth chromosome (88). The deletion is not present in unaffected members of the family, and the genetic material contains the putative familial adenomatous polyposis gene. The polyps result from the inherited absence of the gene. As in the case of retinoblastoma and Wilms' tumor, additional genetic events must occur before carcinoma occurs.

Additional evidence shows that the loss of a gene from chromosome 5 may be one of the genetic events leading to adenocarcinoma of the colon. The results of a study of a series of sporadic carcinomas of the colon reveal that 20 to 40 per cent have a genetic deletion involving this chromosome (89). In this report, no deletions were found in any other chromosome, indicating that the loss on chromosome 5 is a nonrandom loss. The benign adenomatous polyps that were ex-

amined did not have the deletion. Thus, the loss of the gene is a rather late step in the process of developing a sporadic carcinoma of the colon. In addition, because so many sporadic carcinomas of the colon do not have the deletion, the disease may clearly be caused by other genetic mechanisms.

Thus, adenocarcinoma of the colon, retinoblastoma and Wilms' tumor are examples in which the loss of a gene or genes results in cellular transformation. The gene probably codes for a protein that is involved in the negative regulation of growth of cells and its absence results in uncontrolled mitosis. The recessive nature of the transformation in these diseases contrasts that of the better characterized classical oncogenes discussed earlier, which act in a dominant manner.

SUMMARY

The rapid progress in molecular biology has allowed investigators to define some of the basic mechanisms of carcinogenesis. At the molecular level, cellular transformation results from the occurrence of two or three distinct genetic events that uncouple the cell from its normal regulatory mechanisms. One family of genes, the oncogenes, may be particularly important in the process.

The aberrant expression of oncogenes, either by mutation or simply by increased transcription, may result in cellular transformation. The genes usually code for growth factors, growth factor receptors or for proteins involved in the transduction of growth signals into the nucleus. Genetic activation causes the cell to continuously sense a message to undergo mitosis, and the cell no longer responds to its normal regulatory signals.

The concepts are rapidly moving into the clinical realm as the genetic mechanisms of particular neoplasms have been investigated. The neuroblastoma is the first tumor system in which the biologic characteristics of the tumor were found to be related to a known oncogene; the amplification of the *myc* gene is an independent marker of the aggressiveness of the tumor. In addition, much progress has been made in defining the specific genetic lesions responsible for retinoblastomas, Wilms' tumors and carcinomas of the colon that arise in patients with familial polyposis coli. As more knowledge accumulates regarding the exact mechanisms through which the proteins encoded by oncogenes affect these carcinomas and others, it may become possible to design pharmacologic agents rationally to hinder their growth selectively.

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