# Important Advances in Oncology 1986


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# Preface

*Important Advances in Oncology 1986* is the second in a series of annual volumes, each of which will be concerned with the most significant changes in oncologic research and practice that will have taken place during the preceding year. Each year the editors will select approximately 15 areas of research and practice in which change has occurred, with each being described by one or more experts in that particular field. Topics will be chosen for their currency and for their potential effect on the diagnosis, treatment, and prevention of cancer and on an understanding of its origins.

In this second volume, we have selected basic research that describes studies related to human oncogenes; vitamin A analogues in cancer chemoprevention; the role of dietary fiber in cancer prevention; adoptive immunotherapy of cancer using lymphokine-activated killer cells and recombinant interleukin-2; chromosomal rearrangements, genes, and fragile sites in cancer; the role of gene amplification and the reversal of resistance in drug-refractory cancer; and control of gene expression and the replication and pathogenesis of retroviruses.

The clinical progress section contains discussions of the dysplastic nevus syndrome; adjuvant therapy in the treatment of osteosarcoma; epidemiologic patterns of colorectal cancer; cancer imaging with monoclonal antibodies; the therapy of colorectal hepatic metastases; unconventional fractionation schemes in radiotherapy; bone marrow transplantation in the therapy of non-Hodgkin's lymphomas; and interferon in the sequential treatment of hairy cell leukemia.

One of the most important considerations in the selection of topics for this 1986 volume—as in the 1985 volume—was timeliness, and timeliness will continue to be a major consideration in the choice of topics for the volumes to come. All topics will hold promise. The promise of some may not be fulfilled, whereas the promise of others will lead to major change in the way diseases are treated. It is our hope that these volumes will serve to speed such positive and beneficial change.

VINCENT T. DE VITA, JR., M.D.
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Part One

# Basic Research

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Mariano Barbacid

# Human Oncogenes 1

## Introduction ■

During the last 5 years, a series of discoveries have begun to establish the molecular basis of human neoplasia. Oncogenes, thought to exist exclusively in animal tumor viruses (*i.e.*, retroviruses, adenoviruses, papovaviruses, and so forth) have been shown to exist also in a significant fraction of human tumors (for recent reviews, see Refs. 1, 2). Some of the mechanisms by which these human oncogenes acquired their neoplastic properties have been elucidated. Moreover, the function of these human cancer genes is beginning to be unveiled.

To date, only oncogenes belonging to the *ras* and *myc* gene families, as well as c-*abl*, have been clearly implicated in the development of human neoplasia. *ras* oncogenes have been identified in about 15% of the most common forms of human malignancies, such as carcinomas of the lung, colon, and breast, as well as in several types of leukemias and sarcomas. The *myc* gene family has been implicated in various neoplasias, such as Burkitt's lymphoma (c-*myc*), neuroblastomas (N-*myc*), retinoblastomas (N-*myc*), and small cell lung carcinomas (c-*myc*, N-*myc*, and L-*myc*). The c-*abl* oncogene becomes activated by the t(9:22) chromosomal translocation that generates the Philadelphia ($Ph^1$) chromosome, a classic cytologic marker for chronic myelogenous leukemia (CML). Finally, several new human oncogenes have recently been identified either by gene transfer assays or by molecular cloning of the breakpoints of chromosomal translocations specifically associated with certain hematopoietic tumors.

## *ras* Oncogenes ■

### Detection of Transforming Genes by Gene Transfer ■

All mammalian genomes contain certain genes, designated proto-oncogenes, that can acquire cancer-inducing properties upon transduction into the genome of acute transforming retroviruses (for a review, see Ref. 3). The first experimental evidence indicating that human proto-oncogenes might be activated as oncogenes in certain human tumor cell lines was obtained by Weinberg, Cooper, and their respective colleagues. DNAs isolated from two human bladder carcinoma cell lines, EJ and J82 cells, induced malignant transformation of NIH/3T3 mouse cells in gene transfer assays. Shih and co-workers showed that the NIH/3T3 cells transformed with EJ DNA contained human DNA sequences, thus demonstrating the human origin of the oncogene responsible for their malignant phenotype.[5] Similar results were soon obtained with additional human tumor cell lines in Wigler's laboratory[6] as well as in my own.[7] The presence of transforming genes was not limited to established cell lines. Pulciani and associates demonstrated the existence of oncogenes in 5 of 28 human tumor biopsies, including carcinomas and sarcomas, thus demonstrating that oncogene activation was not an artifact of *in vitro* cultivation of human tumor cells.[8] During the past 2 years, many other laboratories have identified oncogenes that can transform NIH/3T3 cells in culture.[1,2] Overall, about 15% of human malignancies possess transforming genes. In some cases, such as acute lymphocytic and myeloid leukemias, the percentage of tumors carrying NIH/3T3-transforming genes may be as high as 50% (Bos H: Personal communication).[9]

### *ras* Gene Family of Oncogenes ■

A relationship between transforming genes present in human tumors and oncogenes transduced by acute transforming retroviruses has been established. In the spring of 1982, three laboratories reported that the oncogene present in T24 and EJ human bladder carcinoma cells was a transforming allele of the human H-*ras*-1 locus.[10-12] The H-*ras*-1 gene is the cellular homologue (proto-oncogene) of the oncogene present in the Harvey strain of murine sarcoma virus and a member of the *ras* gene family.[13] Transforming H-

*ras*-1 genes have been identified in carcinomas[4–7] of the bladder (T24 and EJ cell lines[10–12] and JBT44 tumor[14]), breast (HS242 cell line[15]), lung (HS578T cell line[16]), and kidney (JBT26 tumor[14]), as well as in melanomas (SK2[17] and SK-MEL-146 cell lines[18]). Other transforming genes were found to be alleles of K-*ras*-2,[8,10] the cellular homologue of v-K-*ras*, which is the oncogene of the Kirsten murine sarcoma virus.[13] Transforming K-*ras*-2 genes have been identified in carcinomas of the bladder (A1698 cell line[8]), colon (SW480,[19] A2233,[8] and SK-CO-1[20] cell lines), gall bladder (A1604 cell line[8]), lung (LX-1[10]; A2182,[8] A427,[8] SK-LU-1,[20] Calu-1,[20] PR310,[21] and PR371[21] cell lines and #1615,[8] #1189,[8] and LC10[22] tumors), ovary (OVCA-1 tumor[23]), and pancreas (#1189 tumor[8]), as well as in a rhabdomyosarcoma[8] and in an acute lymphocytic leukemia cell line.[9] A third member of the *ras* gene family of oncogenes was initially described by Shimizu and colleagues in SK-N-SH cells, a neuroblastoma cell line.[20,24] Transforming N-*ras* genes have been identified most often in tumors of hematopoietic origin, including seven acute lymphocytic leukemia cell[8,25]; 1M9 cells, a CML cell line[8]; two primary acute myelogenous leukemias[8,26]; HL-60 cells, a promyelocytic leukemia cell line[27]; and AW Ramos, a Burkitt's lymphoma cell line.[27] Transforming N-*ras* genes have also been identified in carcinomas of the colon,[28] liver,[28] and lung[29]; in four different melanoma cell lines,[18,30] PA-2 cells, a teratocarcinoma cell line[31]; HT-1080 cells, a fibrosarcoma cell line[28,32]; and RD cells, a rhabdomyosarcoma cell line.[32] About 90% of human oncogenes identified by their property of transforming NIH/3T3 cells in gene transfer assays have been found to be members of the *ras* gene family.

## Molecular Structure of Human *ras* Genes ■

As a result of the combined efforts of several laboratories, the molecular structure of the three known human *ras* genes is now known in detail.[33–38] They range in genetic complexity from the simplest, H-*ras*, which is 4.6 kilobase pairs (kbp), to the most complex, K-*ras*-2, which is 40 to 45 kbp.

The human genome contains at least two loci homologous to H-*ras* (H-*ras*-1, H-*ras*-2) and K-*ras* (K-*ras*-1, and K-*ras*-2) genes[39]; however, only H-*ras*-1 and K-*ras*-2 are functional, whereas the other, H-*ras*-2 and K-*ras*-1, correspond to nonfunctional pseudogenes.[35] The use of retroviral and cDNA *ras* probes has allowed the accurate identification of the intron and exon regions of these functional genes. These structural studies have shown that the difference in genetic complexity of human *ras* genes is the result of their different intronic structure; however, H-*ras*-1, K-*ras*-2, and N-*ras* have similar exon sequences.[33–38] In fact, the splice junctions of *ras* genes correspond precisely among all of them, suggesting that they derived from a common ancestral gene that contained a minimum of four exons. K-*ras*-2 shows an additional, diverged fourth exon (IVb) that confers the potential to encode for two alternate products.[36,37]

As a result of their similar genetic structure, the three known *ras* genes code for highly related proteins of 189 amino acid residues, generally designated p21.[40] There are three defined domains of structural significance among the gene products of the known *ras* genes[37,38]: A first domain of virtual identity encompasses the first 80 amino acid residues of p21 proteins; the next 80 amino acid residues define a second domain where the different p21 proteins diverge slightly from each other (85% homology between any pair of H-*ras*-1, N-*ras*, and K-*ras*-2); finally, there is a carboxy-terminal variable region of pronounced divergency. This structural organization suggests that the amino terminal domain has a catalytic or regulatory role that has been conserved in evolution. It is precisely in this region that single amino acid substitutions have been shown to activate the malignant potential of *ras* proteins (see below). The carboxy-terminal variable region may contain the determinants of physiologic specificity for each individual member of the *ras* gene family.

The *ras* genes have been shown to be highly conserved during evolution. Less than 5% variation in amino acids has been found between p21 proteins of human and rat origin (Ruta M: Personal communication).[28] More striking is the high degree of relatedness that the recently identified *ras* genes of invertebrates[41–43] and yeasts[44–47] show with their mammalian counterparts. Although yeast *ras* genes code for larger products (42,000 daltons), they also conform to the three-domain structure defined in the mammalian p21 proteins. In fact, more than 90% of significant amino acids are homologous in the amino-terminal domain in the yeast and mammalian *ras* gene products.[46,47]

## Chromosomal Localization of Human *ras* Genes ■

Techniques of chromosome mapping using human–rodent somatic cell hybrids and *in situ* hybridization to human meiotic chromosomes have allowed the assignment of the human *ras* genes to given chro-

mosomes. N-*ras* has been assigned to the short arm of chromosome 1,[48,49] whereas H-*ras*-1 and K-*ras*-2 have been assigned to the short arms of chromosomes 11 and 12, respectively. H-*ras*-1 has been mapped to 11p15.1-p15.5,[51-53] and K-*ras*-2 has been located to 12p-12.05pter.[49,53,54] The nonfunctional human *ras* homologues have also been mapped: H-*ras*-2 to chromosome X[55] and K-*ras*-1 to 6p23-12.[56] Jahnwar and colleagues,[52] using *in situ* hybridization, have identified K-*ras*-related sequences in the long arm of chromosome 12 and the short arm of chromosome 3. These sequences may represent additional members of the human *ras* gene family; however, this possibility remains to be tested.

A duplication mechanism has been proposed for H-*ras*-1 and K-*ras*-2 loci based on the observation that the short arm of human chromosomes 11 (encoding DLDH A and H-*ras*) and 12 (encoding DLDH B and K-*ras*) shares at least two pairs of genes that probably evolved from common ancestral genes.[57,58] If the duplication-divergence schedule allows for fine tuning of developmental processes, it may follow that each member of the *ras* gene family performs distinct but related functions.

## Mechanism of Malignant Activation of *ras* Oncogenes ■

Comparative analysis of the nucleotide sequences of *ras* oncogenes with their normal cellular counterparts isolated from normal human cells has allowed the identification of the critical change that confers on the *ras* oncogenes their transforming properties.[59-61] In all cases a single point mutation responsible for a single amino acid change in the p21 protein product is responsible for the malignant activation of *ras* oncogenes. Two "hot spots" for activation have been detected thus far: codon 12, located in the first exon,[59-61] and codon 61, located in the second exon.[15,38]

The activated H-*ras*-1 oncogene detected in T24 bladder carcinoma cells differs from its normal counterpart in a single base change (G → T) at codon 12 that results in the substitution of valine for the normal glycine residue.[33,34,59-61] Substitution of glycine by several other amino acids, such as valine, cysteine, arginine, lysine, and aspartic acid, has been observed in several transforming *ras* genes, including H-*ras*-1,[16,33,34,59-63] K-*ras*-2,[21,22,36,37] and N-*ras*.[31,64] Computer-assisted models predict similar conformational changes of the p21 protein products regardless of the residue replacing glycine: A flexible hinge region that allows the amino terminus of normal p21 to fold into the core of the molecule disappears in the transforming p21, leading to a more rigid tertiary structure.[62,66] An exception to this prediction would be the substitution of glycine by proline, a well known $\alpha$-helix breaker. Recently, Seeburg and co-workers have shown that all H-*ras*-1 p21 proteins carrying amino acid residues in position 12 other than glycine and proline retain the ability to transform NIH/3T3 cells.[67] Transforming *ras* genes can also result from mutations at codon 61, leading to the substitution of the normal glutamine residue by several amino acids, such as leucine, histidine, lysine, and arginine.[14,15,17,21,29,38,63,65,68] *In vitro* mutagenesis studies have also shown that substitution of amino acid residues in positions 13, 59, or 63 by certain other residues (*i.e.*, Gly[13] by Asp; Ala[59] by Thr; and Glu[63] by Lys) also leads to the malignant activation of the H-*ras*-1 oncogene.[63] Whether the structural consequences of mutations within these codons are similar to those discussed above remains to be determined.

Some of these activating mutations introduce restriction endonuclease polymorphisms that have been used as molecular markers to identify transforming *ras* genes in human DNAs. For instance, substitution of the first deoxyguanosine of the 12th codon of the human K-*ras*-2 proto-oncogene by a deoxycytidine creates the sequence GAGCTC, which is specifically recognized by Sac I.[22] The first exon of the K-*ras*-2 locus is conveniently located within a 14 kbp Sac I DNA fragment that can easily be identified by Southern blot analysis. The creation of this polymorphic Sac I cleavage site splits the first exon sequences between two Sac I fragments of 5.8 kbp and 8.2 kbp that can easily be distinguished from the single 14 kbp Sac I fragment characteristic of the normal allele.

This restriction fragment length polymorphisms (RFLP) has been successfully utilized in our laboratory to demonstrate the presence of a K-*ras* oncogene in a human lung carcinoma.[22] Examination of DNAs isolated from normal tissue of the same patient, including bronchial and parenchymal cells and blood lymphocytes, showed that none of them contained the activating mutation detected in tumor tissue. Similar results have also been obtained by Fujita and associates using tumors of the urinary tract.[14] These findings indicate that the mutational event responsible for the malignant activation of *ras* genes in human tumors is specifically associated with neoplastic development. Moreover, they unequivocally establish the somatic nature of the activation of *ras* oncogenes.

Increased expression of nonmutant *ras* genes may also lead to malignant transformation. Placing the human H-*ras* proto-oncogene under the control of retroviral enhancer elements confers this proto-on-

cogene with transforming properties in the NIH/3T3 transfection assay.[69] The high levels of expression mediated by the interaction of the proto-oncogene with a retroviral long terminal repeat (LTR) are thought to be responsible for the transformation. In a similar series of experiments we have shown that the normal H-*ras* proto-oncogene can also transform NIH/3T3 cells, providing that multiple copies become stably integrated in the recipient transfected cells.[70]

Increased expression of *ras* genes has also been implicated in the development of naturally occurring malignancies. Amplification of the K-*ras* locus has been reported in a mouse adrenocortical tumor cell line and in a human lung carcinoma biopsy.[70,71] Elevated expression of presumably nonmutated *ras* genes in premalignant as well as malignant tissues has been reported by several authors.[72–75] These observations might be interpreted as evidence for a possible involvement of derepressed *ras* proto-oncogenes in the development of human malignancies, far beyond the 10% to 15% of cases in which transforming (mutated) *ras* genes have been identified by gene transfer assays. Whereas this interpretation should not be ruled out, we must keep in mind that comparably increased *ras* gene expression has been observed in regenerating rat liver.[76,77] In fact, the levels of p21 expression required to transform NIH/3T3 cells[69,70] are far greater than those described above.[72–75] Thus, it is likely that the moderate increase in *ras* gene expression so frequently found in human tumors may be a consequence of the active stage of cell proliferation of the tumor cells as compared with the surrounding normal tissue.

## *ras* Oncogenes in Carcinogen-induced Animal Tumors: A Model to Study the Role of *ras* Oncogenes in Tumor Development ■

A complete understanding of the precise role that *ras* oncogenes play in the development of human neoplasia requires the use of appropriate model systems. The suitability of one such a model has been shown recently in our laboratory.[78,79] H-*ras*-1 oncogenes were specifically and reproducibly activated in mammary carcinomas induced by injection of a single dose of nitroso-methy-urea (NMU) during sexual development of female rats. More than 80% of all tumor DNAs tested, but not DNAs isolated from normal breasts, contained transforming H-*ras*-1 genes as determined by the NIH/3T3 transfection assay.[78,79] These results suggested that the reproducible activation of the H-*ras*-1 locus reflects a specific role of this gene in the growth or differentiation of the target cells in the mammary gland.

Similar results have been obtained in other carcinogen-induced animal tumor systems. Balmain and Pragnell have reported the reproducible activation of the H-*ras* locus in dimethyl benzanthrene (DMBA)-induced mouse skin carcinomas.[80] Interestingly, this oncogene could be identified in premalignant papillomas, suggesting an early role of *ras* activation in tumorigenesis.[81] Guerrero and colleagues have recently reported the specific activation of *ras* genes in mouse thymomas.[82] Consistently, N-*ras* oncogenes were present in NMU-induced tumors, whereas K-*ras*-2 oncogenes were detected when the thymomas were induced by X-rays.[82] Induction of kidney tumors in rats by a single dose of dimethylnitrosomine (DMN) also invokes the reproducible (about 50% of the tumors) and specific activation of the K-*ras* oncogene (Sukumar S: Personal communication; Magee P: Personal communication). These findings are not unique to members of the *ras* gene family. Recently, Weinberg and co-workers have shown the reproducible activation of an EGF-receptor related gene, designated *neu*, in rat neuroblastomas induced by ethylnitroso-urea.[83] Frequent and specific activation of *ras* genes has also been reported in chemically transformed cell lines, including methylcholanthrene-transformed mouse fibroblasts[84] and guinea pig fetal cells mutated either *in vivo* or *in vitro* with four different chemical carcinogens.[85]

Molecular analysis of the transforming H-*ras*-1 genes present in NMU-induced mammary carcinomas revealed that their malignant activation is always due to the same mutational event, a G → A transition in the second nucleotide of the initial 12th codon.[79] G → A transitions are the type of mutation specifically induced by NMU.[86,86a] No such mutations were observed in H-*ras*-1 oncogenes present in mammary carcinomas induced by DMBA, a carcinogen that does not induce specific G → A transitions.[79] These findings have been interpreted as evidence that NMU is the agent directly responsible for the activation of H-*ras*-1 oncogenes, thus representing the first identification of an oncogene as a target for a chemical carcinogen *in vivo*.[79] Another important property of this animal model system is that, because of the high labile nature of NMU, along with the fact that tumors are induced by a single carcinogenic insult, initiation of carcinogenesis can be defined within hours after the administration of NMU. Therefore, if NMU is directly responsible for mutagenesis in the H-*ras* locus, the process of oncogene activation must be concomitant with, and presumably must contribute to,

initiation of carcinogenesis in this animal model system.[79]

Extrapolation of these findings to human cancers should be done with caution. Although the etiology of most human tumors is unknown, it is clear that humans are not exposed to the relatively high dose of carcinogen needed to induce animal tumors. Thus, although *ras* oncogenes are likely to be responsible for the initiation of certain human malignancies, in other cases they may play a role in tumor progression.[18,31]

## Biochemical Properties of the *ras* Gene Products ■

The biochemical role of p21 proteins is being elucidated. Both normal and mutated (transforming) p21 *ras* proteins are located in the inner side of the plasma membrane. They contain tightly bound fatty acids and bind GDP or GTP with equal efficiency.[88-93] Normal p21 molecules also exhibit GTPase activity, an enzymatic function that is either greatly reduced or not present at all in their transforming counterparts.[94-97] Recently, limited sequence homology with the GTP-binding proteins (G proteins) that modulate adenylate cyclase and transducing has been reported by Gilman and co-workers.[98,99] These observations have led to the proposition that *ras* genes may be involved in transducing signals generated by an as-yet-undetermined ligand upon binding to cell surface receptors. If so, the transforming properties of mutated p21 *ras* proteins may result from their inability to hydrolize GTP and, therefore, to modulate signal transduction.

Evidence supporting these ideas has recently been obtained in yeast cells. Yeast possess two *ras* loci designated RAS 1 and RAS 2.[44-47] Although neither of the two is totally indispensible for yeast cells, double mutants in both loci are not viable, indicating that *ras* genes play a vital role in yeast cell proliferation.[100,101] Yeast cells carrying an extra chromosomal *ras* gene in which the glycine residue in position 19 (which corresponds with position 12 of mammalian p21 proteins) has been replaced by valine, exhibit an unusual phenotype that can be characterized by a failure to present a physiologic response sporulation to nutritional stress.[101] As a consequence, yeasts carrying $RAS^{Val19}$ mutant genes are poorly viable in low nutrient, indicating that they require continuous cell division for survival. Similar phenotypic properties have been observed previously in another yeast mutant designated *bcy* 1. This strain has a recessive mutation in the regulatory subunit of the c-AMP-dependent protein kinase; as a consequence, *bcy* 1 cells have constitutively high levels of such kinase activity.[102] Interestingly, *bcy* 1 has been shown to complement RAS $1^-$, RAS $2^-$ mutations, suggesting that RAS loci must be involved in the generation of cAMP.[103] These findings have led Wigler and co-workers to propose that ras proteins, either directly or indirectly, modulate adenylate cyclase activity.[103]

Preliminary evidence suggests that mammalian *ras* p21 proteins, like their yeast counterparts, may also be involved in controlling the cell cycle. Microinjection of bacteria-synthesized p21 molecules into mouse fibroblasts triggers DNA synthesis[104,105]; however, microinjection of monoclonal antibodies against p21 induces the $G_1$ arrest of growing cells.[106] Whatever the specific function of *ras* gene proteins might be, it appears to be intimately associated with the control of cell proliferation.

# c-*myc* and *myc*-Like Oncogenes ■

## c-*myc* Gene and Its Activation by Chromosomal Translocations ■

Identification of oncogenes in human tumors has relied on two basic experimental approaches: identification of transforming genes in gene transfer assays and use of retroviral probes to search for genetic abnormalities in proto-oncogene loci. The latter approach was used in the identification of the c-*myc* locus in the chromosomal translocations characteristic of human Burkitt's lymphoma and mouse plasmacytomas (for reviews, see Refs. 2, 107, 108). Considering the scope of this review, I shall concentrate on studies involving Burkitt's lymphoma.

Burkitt's lymphoma cells are characterized by the reciprocal exchange between the end of the long arm of chromosome 8,[109] which harbors the c-*myc* gene,[110,111] and either of three chromosomes, 14,[112] 2,[113] and 22,[114,115] which carry the immunoglobulin (Ig) heavy chain,[116] λ light chain,[117-119] and κ light chain[119,120] locus, respectively. The t(8:14) translocation is by far the most frequent (90% of the cases) and is characterized by the transfer of the c-*myc* gene (cytogenetically mapped at 8q24) along with the distal end of the long arm of chromosome 8 next to the Ig heavy chain constant region (mapped at 14q32).[110,111,121-125] As a rule, both loci are joined head to head in divergent transcriptional orientations and involve the nonfunctional Ig allele. With the "variant" Burkitt's lymphoma translocations, the c-*myc* locus

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