Oncogenes in Human Cancer: A Review

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Abstract

Mutations in codon 12, 13, or 61 of one of the three ras genes, H-ras, K-ras, and N-ras, convert these genes into active oncoproteins. Rapid assays for the detection of these point mutations have been developed recently and used to investigate the role mutated ras genes play in the pathogenesis of human tumors. It appeared that ras gene mutations can be found in a variety of tumor types, although the incidence varies greatly. The highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%); in thyroid tumors (50%); and in myeloid leukemia (30%). For some tumor types a relationship may exist between the presence of a ras mutation and clinical or histopathological features of the tumor. There is some evidence that environmental agents may be involved in the induction of the mutations.

Introduction

Alterations in the cellular genome affecting the expression or function of genes controlling cell growth and differentiation are considered to be the main cause of cancer. Molecular cancer research aims at identifying the genes that are altered in the various tumor types and elucidating the role of these genes in carcinogenesis. A family of genes that is frequently found to harbor a mutation in human tumors is that of the ras genes. This family consists of three functional genes, H-ras, K-ras, and N-ras, which encode highly similar proteins with molecular weights of 21,000 (1). Mutated ras genes were first identified by their ability to transform NIH/3T3 cells after DNA transfection. Subsequent analysis of a variety of tumor samples revealed that in part of the human tumors one of the three ras genes harbored a point mutation; as a result, the protein product has an altered amino acid at one of the critical positions 12, 13, and 61. The functional and structural resemblance of the ras proteins with the G-proteins controlling adenylate cyclase has led to the proposal that normal p21ras proteins are involved in the transduction of external stimuli, most likely induced by growth factors or factors involved in cell differentiation. The current model is that the ras proteins become activated upon stimulation, transduce the signal to some effector molecule, and subsequently become inactivated. Mutated ras proteins, however, have lost the ability to become inactivated and thus stimulate growth or differentiation autonomously. Despite extensive research, the signals that induce activation of ras proteins and the proteins that are affected by ras proteins in the signal transduction cascade are still unknown. The ras proteins might not be directly linked to cell surface growth factor receptors but might play a more pivotal role in the transduction of several growth or differentiation factor stimuli (1). A protein has been discovered that is involved in the hydrolysis of the GTP bound to ras. This protein, GTPase-activating protein, binds to the effector domain of the ras proteins and might play a role in the transduction of signals from ras further downstream (2-6). Alternatively, other still to be discovered effector proteins might compete with GTPase-activating protein for the effector site (6).

The introduction of new and rapid assay systems for the identification of mutated ras genes has made it possible to analyze large numbers of tumor samples for the presence of ras genes. Recently extensive reviews have appeared about the possible function of ras proteins in both lower and higher eukaryotes (1), about the role chemical mutagens can play in the induction of the mutation (7), and about the presence of mutant ras genes in human tumor cell lines (8).

In this review I will try to summarize our current knowledge about the role mutant ras genes play in human tumors. Other genetic defects of the ras genes, such as amplification or loss of a normal ras allele, have been discussed previously (8).

Detection of Mutated ras Genes

The original assay to identify altered ras genes was based on the ability of the genes to transform the established mouse cell line NIH/3T3 (9-11). This transfection assay was not suitable for the analysis of large numbers of tumors, mainly due to the laborsomeness of the assay. It helped to reveal, however, the positions at which point mutations occur and therefore opened the possibility of analyzing the alterations directly in the tumor DNA. Presently, point mutations are usually detected by selective hybridization with synthetic oligodeoxynucleotide probes, which are specific for the known mutations in codons 12, 13, and 61 of the three ras genes (12, 13). By use of probes specific for each mutation, the exact base pair change can be identified. Alternatively, the position of a mutation can be detected by RNase mismatch cleavage (14). This assay uses an RNA probe which will form a mismatched hybrid with the mutated DNA. RNase A will cleave the RNA at the position of the mismatch resulting in two fragments which can be identified after gel electrophoresis. Both methods make use of tumor DNA, which can be isolated from fresh tumor tissue or from frozen or paraffin-embedded tissue sections and from which the relevant sequences of the three ras genes are amplified in vitro by the polymerase chain reaction (15). Finally, polymerase chain reaction-amplified segments of the ras gene can also be used to determine mutations by sequence analysis (16, 17). A more extensive discussion of the methods currently available to screen tumors for ras mutations has been published previously (8).

Incidence of ras Gene Mutations

The NIH/3T3 assay, in particular in combination with a nude mouse tumorigenicity test (18-20) and the biochemical assays described above, has provided information on the presence and incidence of mutated ras genes in a variety of tumor types. Table 1 gives a summary of these results and deals only with studies in which a significant number of tumors have been analyzed for the presence of mutated ras genes. Information

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1 This work is supported by a grant from the Dutch Cancer Society.

2 The abbreviations used are: p21, M, 21,000 protein; NSCLC, non-small cell lung carcinoma; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphoblastic leukemia; NHL, non-Hodgkin's lymphoma.
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| * n, number of samples tested.  
| * Percentage of samples with a mutated ras gene.  
| * Method used for the detection of mutated ras genes: 3T3, transfection assay with NIH/3T3 cells; ODN, oligodeoxynucleotide hybridization assay; RMC, RNase mismatch cleavage assay; RFLP, restriction fragment length polymorphism.  
| * ras gene preferentially found to be activated.  
| * Only tested for H-ras codon 12.  
| * FAP, familial adenomatous polyposis; IMF, idiopathic myelofibrosis; CML, chronic myeloid leukemia.  
| * Only tested for K-ras.  
| * 13 leiomyosarcomas, 10 histiocytomas, 2 schwannomas, 1 liposarcoma, 1 fibrosarcoma, 1 rhabdomyosarcoma (H. J. van Kranen, personal communication).  
| * See Footnote 5.  
| * See Footnote 3.  
| * See Footnote 6.  

The results presented in Table 1 elicit the following comments.

1. The incidence of mutated ras genes varies strongly among the different tumor types. The highest incidence is found in tumors from the exocrine pancreas, where more than 80% of the tumors harbor a mutated K-ras gene. In carcinomas of the colon and in follicular and undifferentiated carcinomas of the thyroid the incidence is also considerable with one-half of the tumors possessing a mutant ras gene. In several tumor types a mutant gene is found only occasionally, whereas in a number of tumor types no mutated gene has been identified at all. The absence of ras mutations in the latter tumors might be due to the type of target cell from which the tumor arises. In some cells, a mutated ras gene might not be effective because either its level of expression is too low or the mutated ras proteins do not stimulate cell growth. For instance, introduction of a mutated ras gene into a neuronal cell (PC 12) results in differentiation and cessation of cell proliferation (21, 22). Alternatively, a ras mutation in a particular target cell may predispose this cell to a particular tumor type; e.g., a ras mutation in a lung epithelial cell may predispose to adenocarcinoma. This possibility will be discussed later.
In those tumor types in which mutated ras genes have been found in a subset of the tumors, the other tumors do not have such a mutated gene. Yet there are no clear differences between these two subsets. Apparently, other genetic events not involving the ras genes may have the same ultimate effect. What these other events are is still unknown, but whether ras genes are mutated or not can be dependent on environmental factors. For instance, the incidence of mutated ras genes in breast tumors of female rats depends on which chemical mutagen is used for the induction of the tumors. Approximately 90% of the tumors induced by methylnitrosourea have a mutated H-ras gene, whereas tumors induced by dimethylbenz(a)anthracene contain mutated H-ras genes in only 20% of the cases (23).

2. There is some correlation between tumor type and ras gene mutation. In adenocarcinoma of the lung, pancreas, and colon the K-ras gene is the predominantly mutated ras gene, whereas in myeloid leukemia it is the N-ras gene. Some tumor types, like thyroid tumors, seem to lack any specificity. The occurrence of a preponderant mutation of a certain ras gene in a particular tumor type may be related to the fact that various ras proteins have different functions. However, this explanation may be an oversimplification, since there may be no functional difference between the three p21ras proteins when mutated. A mutant ras protein is thought to be in an activated state and independent of whether or not there is an incoming signal. Therefore, if the three mutated ras proteins display any specificity it should be in the interaction with an effector molecule which is to receive the signal from the ras protein. However, the domain which is supposed to bind the postulated effector protein is identical in the three p21ras proteins (24, 25). This suggests that the three ras proteins pass their signals toward the same effector molecule and, consequently, that there is no difference in specificity between the three mutated ras proteins. This hypothesis is supported by the fact that for most tumor types there is no absolute specificity for a mutated ras gene. In vitro DNA transformation experiments also failed to produce any evidence for different specificities of the three mutated ras genes.

If differences in function of the ras proteins are not the determining factor, the observed specificity may be explained by differences in expression. In most tissues all the ras genes are expressed (26–28), but the levels of expression of each of the genes can vary considerably (26). Comparing these levels and the type of ras gene found most frequently mutated do not reveal any correlation. However, the measured expression levels in a tissue does not necessarily reflect the levels in the cell where the ras mutation occurs.

Clinical Aspects

For several tumor types information is available about the stage of tumor development at which the ras gene mutation might have occurred, about the possible specific pathological effects of mutated ras genes, and about the involvement of carcinogenic agents in the induction of the mutations. These tumors will be discussed in more detail.

Colon Carcinomas

Mutational Event. The development of colon carcinomas is considered to pass through at least three stages of progression. The first stage is a small benign tubular type of adenoma or polyp which frequently occurs in the colon of healthy individuals and which does not progress in most cases (29). Occasionally, the tubular type of adenoma increases in size and becomes more villous. This second stage is considered to be more aggressive and frequently contains patches of frank carcinoma tissue. These patches may grow out into the third stage, invasive carcinoma (30). Both in the carcinoma tissue and in the larger, villous type of adenomas mutated ras genes have been detected in one-half of the cases (31–33). Furthermore, in several tumors, the ras mutation was found both in carcinomatous and adenomatous tissue. This implies that the ras mutation occurs before the conversion to malignant carcinoma. In a few cases the mutation was found in the carcinomatous tissue only, indicating that the timing of the mutation is not invariant (32, 33).

In the smaller, tubular type of adenomas the incidence of mutated ras genes was much lower (<20%) (31). The simplest interpretation of this lower incidence is that the mutational event occurs in a cell of the small adenoma which, subsequently, is responsible for the further progression of the disease. This explanation would predict that in some small adenomas the ras mutation is present in only a fraction of the cells. Thus far, the hybridization experiments with oligodeoxynucleotides did not provide evidence for the existence of such a subgroup of cells. An alternative explanation is that the ras mutation is an early or even initial event, which provides some advantage to the adenoma to increase in size and, consequently, to progress into a malignant carcinoma. Detailed analysis of small adenomas should discriminate between these two possibilities.

A low incidence of ras mutations were also observed in the mainly small adenomas from patients with familial adenomatous polyposis, a hereditary predisposition to colon cancer (31, 34). This indicates that with respect to ras mutation this type of familial tumor is similar to the spontaneously occurring colon tumors.

Pathological Features. There is no apparent correlation between the presence of a ras gene mutation in the carcinoma and its anatomical location, depth of invasion, level of differentiation, age, or sex of the patient (31–33). The observation that tumors with a mutation at the second base of K-ras codon 12 are generally more invasive than tumors with a mutation at the first base (33) is not confirmed in other studies.3

Etiology. The mutations found in the K-ras gene are listed in Table 2. It is striking that the majority of the mutations are G-A transitions at the second G of a GG pair. This type of mutation is characteristic for alkylating agents (35) and it is conceivable that these mutagens are responsible for the induction of some of the ras gene mutations in colon tumors.

Cooperating Genetic Defects. ras gene mutations are not the only genetic aberrations found in colon carcinogenesis. Other

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3 J. L. Bos and B. Vogelstein, unpublished observations.
genetic defects that occur frequently are deletions in the short arm of chromosomes 5 (36) and 18 (31) and the long arm of chromosome 17 (37). One intriguing question is whether the deletions can substitute for ras gene mutations and thus occur in tumors that do not contain a ras gene mutation, or whether they cooperate with ras genes and occur exclusively in tumors that do contain a mutant ras gene. Neither seems to be the case. ras gene mutations and chromosomal deletions accumulate independently and in parallel to the clinical progression (31).

**Non-Small Cell Lung Carcinoma**

**Mutational Event.** NSCLC is a group of tumors that includes among others adenocarcinoma, squamous or epidermoid cell carcinoma, and large cell carcinoma. It has been suggested that these tumors are related and originate from a similar precursor cell, the bronchioalveolar epithelial cell (38). In an extensive analysis of these tumors, mutations were found only in adenocarcinoma (39, 40). The incidence is about 30% and nearly all mutations were incurred in K-ras codon 12.

**Pathological Features.** Histologically and clinically, adenocarcinomas with or without a ras mutation do not differ significantly from each other. The carcinomas with a ras mutation seem slightly smaller and metastasize at a slightly later stage, but the number of tumors analyzed is too small to allow a firm conclusion on that point (40).

The relative frequency of mutant ras genes in adenocarcinoma compared to the other NSCLC tumors is intriguing, the more so since all NSCLC may originate from a similar precursor cell. It could be that mutant ras genes are effective only in cells that are differentiated into a secretory cell type. Alternatively, a ras gene mutation in the bronchioalveolar epithelial (stem?) cell may predispose that cell to differentiate into a secreting cell and to become an adenocarcinoma. The latter explanation is supported by the observation that transgenic mice harboring a mutant H-ras transgene under control of either the SV40 early gene promoter or the immunoglobulin enhancer expressed the transgene predominantly in the lung and developed multicentric adenomatous tumors comparable to the well-differentiated adenocarcinoma of the lung in humans (41).

**Etiology.** In adenocarcinoma of the lung, the majority of the mutations in the K-ras gene are G-T transversions, whereas in colon tumors the majority of the mutations are G-A transitions (see Table 2). This difference in mutation spectrum may indicate the involvement of different mutagens in the induction of the ras mutation in lung tumors compared to colon carcinomas. Which mutagens are involved is unknown, but there is some association between the presence of a mutant ras gene in adenocarcinoma of the lung and the smoking history of the patients (40), suggesting that a mutagenic component in tobacco smoke may cause the ras mutation.

**Melanomas**

**Mutational Event.** Melanomas are highly malignant tumors which metastasize very rapidly. The primary tumors are usually small and in some cases occult. The incidence of ras mutations is approximately 20% and the majority of the mutations are found in the N-ras gene. The stage of tumor development at which the ras gene mutation occurs may vary. From one study using cell lines established from different metastatic deposits of one patient it was concluded that the mutation may have occurred during metastasis (42). However, in most cases the mutation is already present in the primary tumor (43). Some primary tumors consist of two cell clones of roughly equal size, each with a different ras mutation. In one of such cases, each of these cell clones was found to have similar metastatic properties (43), suggesting that these two cell clones, except for the ras gene mutation, are genetically similar. Since the two ras gene mutations are in the same region of the N-ras gene, the two cell clones may be descendents of a precursor cell which had incurred DNA damage in the N-ras gene and which, after two rounds of replication, had generated two daughter cells with two different ras mutations. Alternatively, the two cell clones could be independent tumors or independent malignant subclones of a premalignant lesion.

**Pathological Features.** The metastatic properties of melanomas do not seem to be influenced by the presence or absence of mutated ras genes. However, there is a strong correlation between the presence of a ras mutation and a particular localization of the primary tumor. In all cases investigated, the primary tumor with a ras gene mutation was located at a site that is unprotected against sunlight, such as the face or the back of the hand (43).

**Etiology.** The localization of the primary tumors with a ras mutation at sites unprotected against sunlight strongly suggests that UV light is involved in the induction of the ras mutation. This is supported by the type of mutation found. They all occur in or adjacent to dipyrimidine sites, the well known target for UV damage. These data do not exclude a role for UV irradiation in the development of tumors without ras mutations, e.g., the ones located at intermittently exposed body sites; it is conceivable that UV-induced genetic alterations in genes other than N-ras are involved (43).

**Pancreatic Carcinoma**

**Mutational Event.** Tumors of the exocrine pancreas are highly malignant and invariably result in the patient's death. In about 90% of these tumors a mutated K-ras gene has been found and all mutations were located in codon 12 (44, 45). In the remaining 10% of the tumors no mutations in the other ras genes could be detected (45), stressing the very high specificity for the K-ras gene.

**Pathological Features.** The relevance of mutated ras genes in the development of pancreatic tumors was illustrated by transgenic mice which harbor a mutated H-ras transgene under control of either the SV40 early gene promoter or the immunoglobulin enhancer expressed the transgene predominantly in the pancreas-specific elastase I promoter (46). These mice developed neoplasia of the fetal pancreas directly after the onset of elastase gene expression, indicating that the expression of the mutant ras transgene is responsible for the development of the tumor. This result does not necessarily imply that for the induction of pancreatic tumors in humans a single activated ras gene is sufficient. In this case, the ras mutation arises in a single cell surrounded by normal cells which may inhibit the growth of the mutant ras-containing cells (47, 48), whereas in the transgenic mouse model all fetal pancreas cells express the mutant p21 H-ras.

**Etiology.** The mutations found in codon 12 of the K-ras gene have been listed in Table 2. There is a striking difference between the type of mutations found in two different studies. In the first a large fraction of the mutations is a replacement of the first G of the codon by a T (45), whereas in the other the same G is replaced by a C (49). Further studies are necessary to evaluate these differences.

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* R. J. C. Slebos and S. Rodenhuis, personal communication.
**Thyroid Carcinomas**

The tumors of the follicular epithelium of the thyroid gland represent various stages of tumor progression. The benign (micro-) follicular adenomas are considered to progress at low frequency to follicular carcinomas. These carcinomas may progress to the rarely occurring undifferentiated carcinomas. Papillary carcinomas, which are clearly distinguishable from follicular carcinomas, may also progress to undifferentiated carcinomas (50). Mutations in all three ras genes were found both in the benign (micro-) follicular adenomas and in the follicular and undifferentiated carcinomas in one-half of the cases (51, 52). Apparently, the ras mutation can occur early in the development of this tumor type. In papillary carcinomas a low incidence of mutations is observed (51, 53, 54) and no ras mutations were detected in macrofollicular hyperplasias. However, these hyperplasias progress rarely, if at all, into malignant tumors (52).

**Myeloid Disorders**

Mutational Event. Myeloid leukemia comprises a large number of diseases, which can be divided into two main groups: (a) MDS or preleukemia, and (b) AML. MDS is a heterogeneous group of disorders characterized by abnormally low counts of one or more of the blood lineages, combined with bone marrow abnormalities that may evolve into AML in one-third of the cases. By cytogenetic analysis and X-chromosome inactivation studies, it has been shown that the initiating event in this disease most likely affects an early stem cell (55). The subsequent progression of MDS to AML is associated with a gradual increase in blast cells which reflects the outgrowth of a new leukemic cell clone (56). Arbitrarily, patients with more than 30% immature blast cells in their bone marrow are diagnosed as having AML. Of most patients with AML it is unknown whether they have had prior MDS. In about one-third of the MDS and AML patients a mutated ras gene is detected, mostly in the N-ras gene, but also in the K-ras and, infrequently, in the H-ras gene (20, 57–69).

A large amount of information about the occurrence of ras gene mutations in MDS and AML has come from studies in which multiple samples have been analyzed from the same patient. (a) In some MDS patients the mutated ras gene is readily detectable in the bone marrow as well as in the peripheral blood cells, even in the mature T-lymphocytes (59, 69, 70). During complete clinical remission cells with a mutated ras gene may still comprise a major fraction of the bone marrow cells (69). (b) In other patients the mutation is present in only a subpopulation of the bone marrow cells, most likely the leukemic blast cells (61, 63, 69, 71). (c) A mutation present during the initial AML is usually undetectable during clinical remission (61, 69, 71) and during subsequent relapse although in some cases, cells with the mutated ras gene reappear during relapse (68). (d) In one patient the mutation was detected only in the relapse and not in the initial AML (68). (e) In some patients two different mutated ras genes are present presumably in two different cell clones (61). Apparently, with respect to the stage at which ras mutations occur, patients can be divided into at least two groups. The first group has incurred a ras gene mutation in a multipotent stem cell and therefore the mutation is present in all bone marrow cells as well as in the mature peripheral blood cells (69). The second group of patients has obtained the ras mutation later in the course of the disease and the cells harboring a ras mutation represent a newly evolved leukemic cell clone (71). This newly evolved cell clone may mark the evolution from MDS to AML but may also represent the evolution of a new, more malignant cell clone later in the course of the disease or after clinical remission. Whether in these cases the mutation occurs in an already affected early stem cell or in a more committed cell is still unknown. Dependent on the sensitivity of the mutant ras-containing cell clone for chemotherapy, cells with a ras mutation may disappear completely or only partly during chemotherapy. In the latter case the mutation may reappear in a subsequent relapse (68, 69, 71). Mutant ras-containing cell clones in patients with a mutation in a multipotent stem cell appear to be more resistant to chemotherapy than cell clones where the mutations had occurred later in the course of the disease (69).

Pathological Features. A striking observation is that the ras mutation is preferentially found in AML classified as M4 (myelomonocytic) or M5 (monocytic) (61, 68, 69) and MDS classified as chronic myelomonocytic leukemia (65) or classified differently but having monocytosis (69). This suggests that the myelomonocytic or monocytic cell differentiation is preferentially affected by a mutant ras gene. In some of these cases the ras mutation is present in the multipotent stem cell, indicating that the ras mutation affects only the maturation of the myelomonocytic cells and not the maturation of other cells like lymphocytes and erythrocytes. It is not clear, however, whether a given ras mutation preferentially influences hematopoiesis towards an abnormal myelomonocytic differentiation or whether it provides a preferential growth advantage to an MDS with an abnormal monocytic cell lineage. Furthermore, it is unknown whether mutated ras on its own can perform these properties.

In MDS and AML the presence of ras mutations might have some direct clinical relevance. (a) As pointed out by several investigators MDS patients with a ras gene mutation may have a higher chance to progress into AML and thus may have a poorer prognosis (63–65, 69). The prognostic value of ras mutations may be limited, however, since several MDS patients with a ras mutation, including patients with a mutation in the multipotent stem cell, have a stable disease (65, 67), indicating that a ras mutation is not a prognostic factor on its own. Similarly, AML patients with a ras mutation do not have a prognosis different from that of patients without one. (b) The ras mutation might be a suitable marker for monitoring the effect of chemotherapy and detecting minimal residual disease (61, 69, 72, 73).

Etiology. The types of mutations found in AML and MDS are presented in Table 3. The majority of the mutations are present in codon 12 or 13 and one-half of these mutations represent the substitution of an A residue for the second G of a GG pair. As mentioned for colon carcinomas, this type of mutation is typical for alkylating agents. However, only one ras gene mutation, a G-T transversion in codon 13 of the N-ras gene, has been found in 13 patients with an AML or MDS related to therapy with alkylating agents (66).

**Lymphoid Malignancies**

Mutational Event. Lymphoid malignancies comprise a large variety of diseases, including ALL, CLL, and NHL. From the analysis of cell surface markers and rearrangements in immunoglobulin genes or T-cell receptor genes it is known that ALL
involve cells of an early differentiation grade whereas CLL and NHL probably originate from a more differentiated precursor cell. ras gene mutations have been detected in only a low percentage of patients with ALL, preferentially in the non-B, non-T ALL (62, 74, 75) and hardly, if at all, in patients with CLL and NHL (75). This may indicate that lymphoid cells, in particular the more differentiated ones, are refractile to either the mutational event or the effect of mutant ras genes. This latter possibility is supported by the above described observation that a mutant ras gene can be present in apparently normal lymphocytes from patients with MDS. In some of the ALL patients the mutation is present in only a subfraction of the bone marrow cells, notwithstanding the fact that 90% of these bone marrow cells consist of leukemic blast cells. This may indicate that a new cell clone with a mutant ras gene is evolving or, alternatively, that the mutant ras clone is a residual cell clone that has been overgrown by cells not harboring a mutant ras gene (75). Follow-up studies may discriminate between these two possibilities.

**General Conclusions**

The rapid biochemical assays for ras gene mutations have provided an overwhelming amount of information about the presence of ras gene mutations in human tumors. For some tumor types, ras gene mutations have been found in either a small or a large percentage of the tumors; in these tumors, the mutated ras gene product probably plays a role in the development of the tumor. From the present data the picture emerges that tumor types with mutated ras genes most frequently are hematological malignancies of the myelomonocytic lineage and carcinomas, in particular adenocarcinomas, whereas tumor types in which ras gene mutations do not seem to play an important role are tumors of neuroectodermal origin and dif-

**Table 3 ras mutation found in AML and MDS**

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* Data compiled from Refs. 20, 57, 59-61, 63, 65, and 67-69 and Footnote 3.

**References**


* J. J. Yunis and J. L. Bos, unpublished observations.


