Activated ras Oncogenes in Human Thyroid Cancers

Nick R. Lemoine, Edward S. Mayall, Fiona S. Wyllie, Christine J. Farr, David Hughes, Rose Anne Padua, Valerie Thurston, E. Dilwyn Williams, and David Wynford-Thomas

INTRODUCTION

Activated oncogenes of the ras family have been identified in a wide range of solid and hematological malignancies, but usually in only about 10 to 15% of cases of randomly selected tumors (1). There is some evidence to suggest a specificity of activation of one oncogene in a particular tumor type (notably H-ras in urinary tract tumors, K-ras in colon and lung cancers, and N-ras in hematological neoplasms; see Ref. 1 for review), and in some experimental model systems there are definite associations of certain ras oncogene mutations with individual etiological agents, including chemical carcinogens and ionizing radiation (1).

Although thyroid carcinoma is a relatively uncommon clinical malignancy, it has special interest for two reasons. (a) A single epithelial cell type (the thyroid follicular cell) may give rise to two malignant differentiated tumor types (follicular carcinoma and papillary carcinoma) with very different pathological features and clinical behavior. (b) Papillary thyroid cancer has a well-known association with previous accidental or therapeutic radiation exposure. We therefore investigated the possibility that the marked contrast in tumor characteristics might be associated with differences in oncogene activation, using the focus-induction and nude mouse tumorigenicity assays to search for dominant transforming genes, including those of the ras family. [We previously published a brief preliminary report of transforming activity due to activated H-ras in 2 cases of follicular thyroid cancer (2).]

RESULTS

DNAs prepared from the 5 follicular carcinomas (HFC1-5) and 10 papillary cancers (HPC1-10) were examined for their abilities to transform NIH3T3 cells, using both the focus-induction assay (3) as well as the cotransfection/nude mouse tumorigenicity assay (4, 5). The results are shown in Table 1.

DNA from only one tumor (HFC2) was capable of inducing foci of morphologically transformed cells in first round transfection, with an efficiency of 0.01 focus/μg of genomic DNA. In second and third rounds of transfection all DNAs tested were capable of inducing foci at higher efficiencies (0.05 to 1.2 foci/μg of DNA).

DNAs from 5 of 5 follicular tumors and 9 of 10 papillary tumors registered in the nude mouse tumorigenicity assay in the first round of transfection, with tumors appearing within 6 wk of injection of 10⁷ pooled cells. In the second and third rounds of transfection the latent interval to tumor appearance was reduced. DNA prepared from normal human leukocytes and tested in parallel control assays registered only a very low incidence of tumors (1 of 20) which appeared more than 10 wk after injection.

Southern blot analysis of first round nude mouse tumors with specific probes for oncogenes of the ras family showed that the
ras ONCOGENES IN HUMAN THYROID CANCERS

Table 1 Transfection analysis of malignant human thyroid tumors

<table>
<thead>
<tr>
<th>Specimen code</th>
<th>DNA source</th>
<th>1st round</th>
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<tr>
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<td>0/10</td>
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<tr>
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<td>Lymph-nodal metastasis of papillary Ca</td>
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* FA, focus induction assay, results expressed as (number of foci)/(number of dishes of 1.5 x 10^6 NIH3T3 cells transfected); NMTA, nude mouse tumorigenicity assay, results expressed as (number of tumors)/(number of sites injected with pooled genetin-resistant colonies from 2 or 3 dishes, approximately 5 x 10^6 cells); Ca, carcinoma.

Fig. 1. Detection of human Ha-ras sequences in first round transfectants. Ten μg of each DNA were digested with BamHI, electrophoresed in 0.7% agarose, and transferred to a nylon filter. Hybridization was carried out with the insert of pEJ (7) which is specific for human Ha-ras. wbc, normal human leukocyte DNA; NIH, NIH3T3 DNA; FC2, FC3, first round transfectant DNAs obtained after transfection of genomic DNA from these follicular cancers (Table 1). Transforming activity was due to human K-ras in 2 cases (HFC2, HFC4), human N-ras in 2 cases (HFC4, HPC10), and human H-ras in 2 cases (HFC1, HFC3) (Figs. 1 to 3). These results indicate that 80% of follicular cancers analyzed possessed an activated ras oncogene, in contrast to only 20% of papillary cancers (Table 2).

No signal was detected with ras oncogene probes in the DNA of nude mouse tumors derived from transfection of DNA from the other 9 cases. Hybridizations of the DNA from second and third round nude mouse tumors from these cases with the

Fig. 2. Detection of human N-ras sequences in first round transfectants. Ten μg of each DNA were digested with EcoRI, electrophoresed in 0.7% agarose, and transferred to a nylon filter. Hybridization was carried out with the insert of pNR800 (8) which is specific for human N-ras. wbc, normal human leukocyte DNA; NIH, NIH3T3 DNA; FC4, PC10, first round transfectant DNAs obtained after transfection of genomic DNA from a follicular cancer (FC4) and a papillary cancer (PC10); see Table 1.

Fig. 3. Detection of human Ki-ras sequences in first round transfectants. Ten μg of each DNA were digested with EcoRI, electrophoresed in 0.7% agarose, and transferred to a nylon filter. Hybridization was carried out with a human Ki-ras intron probe (9), wbc, normal human leukocyte DNA; NIH, NIH3T3 DNA; FC2, PC4, first round transfectant DNAs obtained after transfection of genomic DNA from a follicular cancer (FC2) and a papillary cancer (PC4); see Table 1.

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Table 2 Transforming genes in human thyroid cancer

<table>
<thead>
<tr>
<th>Transforming activity</th>
<th>Follicular carcinoma</th>
<th>Papillary carcinoma</th>
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<td>Activated ras genes, total</td>
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<td></td>
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<tr>
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<td>2/10</td>
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<td>H-ras 61 arg</td>
<td>H-ras 61 arg</td>
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</tr>
<tr>
<td>N-ras mutants</td>
<td>N-ras 61 arg</td>
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</tr>
<tr>
<td>K-ras mutants</td>
<td>K-ras 12 ser</td>
<td>K-ras 13 asp</td>
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</table>

Fig. 4. Slot-blot analysis for mutations of human H-ras at codons 12 and 61. Only the filters that showed specific hybridization at the discriminating temperature are illustrated. In addition to the normal H-ras allele present in each of the primary tumors HFC1 and HFC3, there is also an abnormal H-ras allele with mutation at codon 61 substituting arginine in place of glutamine. The activated human H-ras oncogenes present in NIH3T3 transfectants (HFC1 Nu1 and HFC3 Nu1) derived from these cases possess an identical mutation to that present in the primary tumor. CON, normal leucocyte DNA. H12 primers, CTGAGGAGCGATGCGAGGAAAT and AGTGGGGTCGTATTCGTCCA; H61 primers, GTCATTTTGAGGAGACGTTG and ACACACAGGAGCCCTCC; H12 glycine (wild type), TACTTCTCTGGCCGGCGGT (antisense); H61 arginine, TACTCCTCGGGCCGGCGGT (antisense).

Fig. 5. Slot-blot analysis for mutations of human N-ras at codons 12 and 61. In addition to the normal N-ras allele present in each of the primary tumors HFC4 and HPC10, there is also an abnormal N-ras allele with mutation at codon 61 substituting arginine in place of glutamine. The activated human N-ras oncogenes present in NIH3T3 transfectants (HPC4 Nu1 and HPC10 Nu1) derived from these cases possess an identical mutation to that present in the primary tumor. CON, normal leucocyte DNA. N12/13 primers, CTGTTGTGAGATTGACTGAGT and GGTGGGATCATATTCTA; N61 primers, GTTTAGATGGGAAACCTG and ATACACAGGAGCCCTCCG; N12 glycine (wild type), GGAGCCAGGTTGTTGGGAA (sense); N61 glutamine (wild type), ACAGCTGGAGAAAGAGTA (sense); N61 arginine, ACAGCTGGAGAAAGAGTA (sense).

DISCUSSION

The detection of serially transmissible transforming activity in 100% of the follicular cancer DNAs and 90% of the papillary cancer DNAs studied was an unexpected finding, since previous studies have shown only 10 to 20% of randomly selected human tumors to contain transforming genes detectable by transfection assays (13, 14). The only previous report of oncogene activation in human thyroid cancer (15) analyzed solely papillary cancers and found a lower frequency of transforming activity (25%) than that shown in our study of these tumors (90%). Those authors conclude that the transforming activity could be ascribed to a putative novel oncogene in human papillary cancer. Our finding of a higher rate of transforming activity is most likely explained by the greater sensitivity of the tumorigenesis assay, as reported previously (5). Indeed, only one of the transforming genes in our tumors (the mutant K-ras allele of HFC2) was detectable by the focus induction assay.

In most transfection studies of human tumor material, transforming activity has been found to be due to a member of the ras oncogene family (for review see Ref. 1). Activated K-ras oncogenes have been found in tumor tissue from a wide range of neoplasms (but especially carcinomas of lung and colon) and activated N-ras oncogenes most often in hematological malignancies; in contrast, activated H-ras can be found in only a relatively small number of carcinomas (notably bladder cancer (16) and melanoma cell lines (17). It is possible, however, that the true frequency of ras oncogene activation (particularly K-ras activation) is underestimated by transfection studies, since...
in a parallel analysis of colonic cancers RNase A mismatch cleavage demonstrated mutant K-ras genes in 39% (26 of 66), while transforming activity was detected in only 20% (6 of 30) by the focus induction assay (18).

Our finding of a high frequency of ras oncogene activation in follicular cancers (80%) is made more intriguing by two other observations. (a) Activated alleles of all three ras oncogenes (H-ras, K-ras, and N-ras) were found in this tumor type, which we believe is a novel finding since to our knowledge, activation of all three members of the ras oncogene family has never been reported in tissue samples (as opposed to derived cell lines) from a single tumor type. (b) There appears to be a preferential mechanism of activation, namely, replacement of glutamine by arginine at position 61 in all of our H-ras or N-ras samples. Previously this mutation has been reported sporadically, for instance, in two urinatry tract tumors (16, 19) and in a proportion of cases of acute myeloid leukemia (20–22). Mutation of K-ras to give serine at position 12 has been reported in carcinomas of the stomach and colon (19, 23). Mutation of K-ras to give aspartic acid at position 13 had recently been described in bone marrow cells of a patient with myelodysplastic syndrome (24) and in the breast cancer line MDA-MB231 (25).

The relative strength of signal detected after hybridization of replicate filters with wild-type and mutant specific probes suggests that, in most of our cases, there are fewer copies of the mutant ras oncogene than of the normal ras allele in the DNA of the primary tumors. Of course, a normal allele is present in all tumor cells that are heterozygous for the mutant gene, and uninvolved stromal cells will have two copies of the normal gene. However, in one case (HFC 3) the signal with mutant probe is stronger than that with wild-type probe, suggesting that the activated oncogene is present in slightly greater copy number than the normal allele. In this study it was not possible to assess the proportion of tumor cells which possess the activated gene, since DNA was extracted from homogenized tumor tissue, but we are carrying out a more detailed investigation of other tumors (using DNA extracted from tissue sections) to correlate histopathological features with ras mutations.

Our finding of a marked difference in the frequency of activated ras oncogenes detectable by transfection assays in papillary cancers (20%) compared with follicular cancers (80%) suggests a connection between this pattern of oncogene activation and the marked difference in biological behavior of these tumor types, both derived from the follicular epithelial cell. Papillary carcinoma is typically multifocal and not encapsulated, invades the lymphatics, and metastasizes to the local lymph nodes. Follicular carcinoma is typically solitary and encapsulated, invades veins, and when it metastasizes often involves bones and lungs (26).

Transfection experiments have shown that the metastatic potential of tumor cells may be modified by introduction of an activated ras oncogene, and it has been suggested that ras gene product may trigger a program of metastasis that is specific for the particular cell type (27, 28). Transfection of an activated H-ras oncogene enhanced the spontaneous metastasis of MT1C1.5/7 mouse mammary carcinoma cells (29) and also induced the spontaneous metastasis of SP1 mouse mammary carcinoma cells (30), after s.c. inoculation in nude mice (the predominant target for metastasis in both these studies was the lungs). This preferential hematogenous metastasis after introduction of the activated ras oncogene into carcinoma cells has much in common with the almost exclusively hematogenous metastasis of follicular thyroid cancers in which we have identified a very high frequency of ras oncogene activation.

REFERENCES

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