Activated ras Oncogenes in Human Thyroid Cancers

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ABSTRACT

Human thyroid epithelial (follicular) cells give rise to two malignant tumors—"follicular" carcinomas, which metastasize almost exclusively via the bloodstream, and "papillary" carcinomas, which metastasize predominantly via lymphatics (Williams, E. D. In: W. Duncan (ed.), Recent Results in Cancer Research: Thyroid Cancer, pp. 47–55. Berlin: Springer-Verlag, 1980). We have investigated whether this contrast in biological behavior might be associated with different patterns of oncogene activation. DNA transfection analysis of five follicular and ten papillary carcinomas indeed showed a statistically significant difference in the pattern of genes responsible, activated ras oncogenes being found in 80% of follicular tumors but only 20% of papillary tumors. In addition, in follicular cancers we have found activation of all three ras oncogenes (H-ras, K-ras, and N-ras), the first time that this has been demonstrated in a primary human tumor type (as opposed to cell lines). We suggest therefore that ras activation may be an important determinant of metastatic capability in these epithelial cancers.

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 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).]

MATERIALS AND METHODS

Tumor Material. The human tumor tissue analyzed in this study was mostly obtained from surgical specimens removed at the University Hospital of Wales in Cardiff, but cases were also kindly provided by Dr. P. A. Hall of St. Bartholomew's Hospital in London, United Kingdom, and C. A. Pegg of City Hospital in Nottingham, United Kingdom. One patient contributed both a follicular cancer (HFC4) and a papillary cancer (HPC9) which had been surgically removed on different occasions. Brief details are provided in Table 1.

Transfection Assays. The focus induction assay was performed by the protocol which we have previously described (3). The tumorigenicity assay, which depends upon in vivo selection of transformed cells (4), was performed essentially as described by Fasano et al. (5) using cotransfection of the dominant drug-resistant selectable marker pSV2Neo (6) followed by G418 selection. Nude mice given s.c. injections of pooled cells were observed for 3 mo.

Southern Blot Analysis. This was performed as previously described (7), using nylon filters (Hybond; Amersham International). Human repetitive sequences were detected with the 0.3-kilobase insert of BACRNA3 (8), labeled by a random primed method (or nick-translated genomic DNA previously sonicated to average size 4 kilobases, in which case the filter was prehybridized with 50 μg/ml of NIH 3T3 DNA, and the hybridization mixture also included 25 μg/ml of NIH 3T3 DNA). Human H-ras was detected using the 6.6-kilobase insert of pEJ (9). Human N-ras was detected using the insert of pN8R800 (a 0.8-kilobase EcoRI/SstI fragment derived from pAT8.8; Ref. 10) kindly provided by Dr. C. J. Marshall, Institute of Cancer Research, London, United Kingdom. Human K-ras was detected using the insert of the Amprobe K-ras, intron probe (obtained from Amersham International) which is equivalent to the insert of p640 (11).

Polymerase Chain Reaction Amplification and Oligonucleotide Probing. Selective amplification of 60- to 130-base pair DNA fragments surrounding the coding region of interest in each of the transfected ras genes in first round nude mouse tumors was achieved using the polymerase chain reaction, and oligonucleotide probing performed essentially as these techniques are described in Ref. 12.

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 10³ 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
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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<th>3rd round</th>
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<td>FA NMTA</td>
<td>FA NMTA</td>
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<td>HPC 3</td>
<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 geneticin-resistant colonies from 2 or 3 dishes, approximately 5 x 10^6 cells); Ca, carcinoma.

Table 1 shows the transfection analysis of malignant human thyroid tumors. The transforming activity was due to human K-ras in 2 cases (HFC2, HPC4), human N-ras in 2 cases (HPC4, 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 tumours derived from transfection of DNA from the other 9 cases. Hybridizations of the DNA from second and third round nude mouse tumours from these cases with the

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 a follicular cancer (FC2) and a papillary cancer (PC3); see Table 1.

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.
Table 2 Transforming genes in human thyroid cancer

<table>
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<th>Transforming activity</th>
<th>Follicular carcinoma</th>
<th>Papillary carcinoma</th>
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<tbody>
<tr>
<td>Activated ras genes, total</td>
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<td>2/10</td>
</tr>
<tr>
<td>H-ras mutants</td>
<td>H-ras 61 arg</td>
<td>61 arg</td>
</tr>
<tr>
<td>N-ras mutants</td>
<td>N-ras 61 arg</td>
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<tr>
<td>K-ras mutants</td>
<td>K-ras 12 ser</td>
<td>K-ras 13 asp</td>
</tr>
</tbody>
</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, NGAGCGATGACGGAAT (antisense) and GGTGGGGTCGTATTCGTCCA; H61 primers, CTGGTGTGAAATCCGCCGG (sense) and GGAGCAGGTGGTGTTGGGAA (sense); HPC4 had a codon 13 mutation (G to A transition in the second nucleotide) that substitutes aspartic acid for glycine at this position.

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, CTGGTGTGAAATGACTGAG and GGTGGGGACATATTTCTCTA; N61 primers, GTTATAGTGGAACCTG and ATACAGAAGGAGCCTCG; N12 glycine (wild type), GAGACAGTGTTGTGGGAA (sense); N61 glutamine (wild type), ACACCTGAGCAGAAGAGTA (sense); N61 arginine, ACACCTGAGCAGAAGAGTA (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

BLUR8 probe (9), and similarly with labeled genomic DNA, show the presence of human repetitive sequences (data not shown), but the genes responsible have not yet been identified.

In order to identify the mutation responsible for the transforming activity in the cases where human ras oncogenes were detected in transfection assays, we used a panel of oligonucleotide probes specific for all possible mutations at codons 12 and 61 of the human H-ras, K-ras, and N-ras genes, and also at codon 13 of human K-ras and N-ras. In each case the presence of the activating mutation of the relevant ras oncogene was confirmed in the original primary thyroid tumor DNA and the nude mouse tumors derived by transfection (Table 2). As shown in Figs. 4 and 5, mutation at codon 61 was detected in the H-ras genes transfected from both HFC1 and HFC3 and in the N-ras genes transfected from HPC4 and HPC10. In all these cases the mutation was an A to G transition in the second nucleotide of the codon, resulting in arginine at position 61 instead of the normal glutamine. In the two cases involving K-ras, different mutations were identified (see Fig. 6): the K-ras allele derived from HFC2 possessed a codon 12 mutation (G to A transition in the first nucleotide) resulting in serine instead of glycine at this position, while the K-ras allele derived from
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 ras, K-ras, and N-ras has never been identified a very high frequency of ras oncogene activation. 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 MTIC 1.5/7 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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