Detection of Point Mutations in N-ras and K-ras Genes of Human Embryonal Rhabdomyosarcomas Using Oligonucleotide Probes and the Polymerase Chain Reaction

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INTRODUCTION

In recent years recombinant DNA technology has allowed the isolation and characterization of several genes which are implicated in oncogenesis. For example, transfection of tumor DNA into NIH3T3 fibroblasts has revealed activated transforming genes in 10–20% of transformed cell lines and solid tumors (1–3). Most of these were either H-, K-, or N-ras, members of a gene family that had previously been encountered as oncogenes in acutely transforming retroviruses (4–8). Sequence analysis of transforming ras genes from human tumors showed that point mutations at codons 12, 13, or 61 commonly confer transforming activity upon the p21 ras gene products (see Ref. 9 for review). Point mutations introduced into other regions such as codons 59, 63, and 116 can also induce transformation in experimental models but these mutations have not yet been detected in human tumors (10, 11).

Although several oncogenes have been detected by this approach, the NIH3T3 transfection assay has several disadvantages as a method for investigating the epidemiology of ras gene activation in human tumors. Large amounts of high molecular weight DNA are required which may be difficult to obtain from substantial numbers of rare or small tumors. Moreover the technique is time consuming, labor intensive, and can be capricious in execution. Recently, however, technical methods have become available which require only limited amounts of DNA and which allow examination of large numbers of tumor specimens including material from histopathology archives. One of these, the RNase protection assay, detects single base pair mismatches between RNA/RNA or RNA/DNA hybrids formed in vitro from cloned ras gene segments and cognate sequences from tumors (12). Alternatively, DNA from tumors may be hybridized under stringent conditions to a panel of oligonucleotide probes each of which is complementary to a mutated homologue of codons 12, 13, or 61 of the ras genes (13). The sensitivity of both methods may be increased by amplifying the copy number of the target sequences several thousand fold using the polymerase chain reaction (14).

The use of the RNase protection assay and oligonucleotide probes has clarified the patterns and incidence of ras gene mutations in human tumors. Activated ras genes are found in both epithelial and hemopoietic tumors. For example most pancreatic carcinomas, approximately 50% of colon tumors, and 20–30% of cases of acute myeloid leukemia contain mutated ras genes (12, 15–18). However in some tumors such as carcinomas of the breast or ovary, ras gene activation appears to be a rare event (19, 20). The relevance of these differences to etiology or tumor biology remain unclear.

There remain, however, a number of other major tumor categories in which the role of ras gene mutation has not been determined. In particular there is little published data concerning the sarcomas, malignant tumors which recapitulate the differentiation of mesenchymal tissues such as fat, connective tissue, bone, and cartilage. Classified within this group are the rhabdomyosarcomas which are tumors differentiating towards striated muscle (21). Transfection of rhabdomyosarcoma DNA into NIH3T3 cells has previously demonstrated the presence of transforming homologues of N-ras and K-ras in a rhabdomyosarcoma cell line and primary tumor, respectively (1, 22). Using the polymerase chain reaction and oligonucleotide probes corresponding to wild type and mutant ras sequences we have therefore searched for ras gene mutations in a series of rhabdomyosarcomas, most of which were derived from paraffin-embedded histopathological material.

MATERIALS AND METHODS

Extraction of DNA from Paraffin-embedded Specimens. Rhabdomyosarcoma specimens were obtained mainly in the form of paraffin blocks from histopathology archives except in two cases where frozen tumors were available. Tumors were obtained from the Royal Marsden Hospitals, London and Surrey, St. Thomas’ Hospital, London, Kingston Hospital, All Saints Hospital, Chatham, and West Wales General Hospital. The diagnosis of rhabdomyosarcoma was made by at least two pathologists on light microscopy of standard hematoxylin & eosin-stained sections and in most cases was confirmed by immunochemistry and/or electron microscopy. DNA was extracted from histopathology archive material using a previously described method with minor modifications (23). 25- x 20-μm sections were cut from paraffin blocks of formalin-fixed rhabdomyosarcoma specimens and incubated at 48°C in 1 ml of extraction buffer [100 mM Tris-HCl (pH 7.5), 40 mM NaCl, 10 mM EDTA] to which was added 100 μl 10% (w/v) SDS1 and 20 μl of a 25 mg/ml solution of proteinase K. After 16–24 h a further 0.5 ml of extraction buffer, 50 μl of SDS and 10 μl of proteinase K were added and the extraction continued for 24 h. The solution was

1 The abbreviations used are: SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; TAQ buffer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% (w/v) gelatin; SSC, standard saline citrate (0.15 M sodium chloride/0.015 M sodium citrate, pH 7.4).

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2 MRC Training Fellow.
twice extracted with phenol, and subsequently with a 1:1 (v/v) mixture of phenol:chloroform and a 25:1 mixture of chloroform:isoamyl alcohol. After addition of sodium acetate to a final concentration of 0.3 M, nucleic acids were precipitated at −20°C for 16–24 h by addition of two volumes of ethanol. The precipitate was pelleted and redissolved in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA). The concentration of DNA was measured using a fluorometer.

PCR. 1 μg of DNA was made up to 97 μl of a solution containing TAQ buffer, 200 μM dATP, dGTP, dCTP, and dTTP and 1 μM each primer in a sterile 0.5 ml Eppendorf tube which was then heated at 94°C for 10 min. Three μl of TAQ buffer containing three units of TAQ polymerase (Cetus Corporation) were added and the whole mixture was overlayed with 100 μl of paraffin oil to prevent evaporation. The DNA was subjected to 40 cycles of the PCR on a Cetus/Perkin-Elmer DNA Thermal Cycler. Each cycle was composed of three phases; 55°C (annealing), 72°C (extension), and 94°C (denaturation) each for 2 min. The success of the PCR was assessed by running 10 μl of the final mixture on a 2% (w/v) agarose gel and visualizing the product as a 100–130 base pair band by staining with ethidium bromide. The PCR primers were 20mer oligonucleotides complementary to sequences flanking codons 12 and 61 of H-, K-, and N-ras.

Hybridization to Oligonucleotide Probes. Ten μl of the total PCR mixture was dotted onto three duplicate nylon filters (Hybond N, Amerham International) using a dot blotting apparatus. The filters were prehybridized by immersing the filters in 0.5 M NaOH, 1.5 M NaCl for 2 min, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), for 1 min, dried and fixed to the membrane by exposure to UV light for 3 min. Filters were prehybridized for 30 min at 55°C in 3 mM tetramethyl ammonium chloride, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.3% w/v SDS, 5X Denhardt’s solution (100X Denhardt’s is 2% w/v polyvinylpyrrolidone, 2% w/v bovine serum albumin, 2% w/v Ficoll) and 100 μg/ml sonicated, denatured, salmon testes DNA. Hybridization to radiolabeled oligonucleotide probes was carried out in a fresh sample of the same solution at the same temperature for 1 h. The filters were rinsed twice in 2X SSC at room temperature and washed for 30 min in 3 mM tetramethyl ammonium chloride, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.3% w/v SDS. The temperature of the wash varied from 59 to 65°C depending on the oligonucleotide probe used. Filters were exposed to autoradiographic film at −70°C for periods ranging from 1 to 16 h. The probes used were 20mer oligonucleotides (except in the cases of K-ras codons 12 and 13 which were 19mers) complementary to wild type and mutant sequences at H-ras codons 12 and 61, K-ras codons 12, 13, and 61, and N-ras codons 12, 13, and 61. Five pm of each oligonucleotide probe was end labeled with γ-32P]dATP using T4 polynucleotide kinase and purified over Nensorb 20 columns. The unlabeled probes and PCR primers were generously donated by Dr. C. J. Marshall (Institute of Cancer Research). To confirm the presence of a ras gene mutation in a tumor, the PCR was repeated on a further aliquot of tumor DNA, the product run on a 2% w/v agarose gel and blotted onto nylon filters by capillary transfer in 20X SSC. Hybridization to oligonucleotide probes was carried out as described above.

RESULTS

The histopathology and the clinical details of the 21 cases of rhabdomyosarcoma used in this investigation are shown in Table 1. Most fall into the category of embryonal rhabdomyosarcoma, consistent with the higher frequency in the general population of this subtype compared to alveolar and pleomorphic tumors.

Up to 200 μg of DNA was extracted from the paraffin-embedded material and this showed the extensive degradation characteristic of DNA from this source (data not shown). DNAs were subsequently subjected to the PCR in order to amplify short DNA segments including codons 12 and 61 of the three ras genes. The success of the PCR was assessed by the presence of a 100–130 base pair ethidium bromide staining band on 2% (w/v) agarose gels or the presence of a signal when hybridized to a wild type probe. All but four of the DNAs amplified efficiently in the PCR and since these failed irrespective of the priming oligonucleotides used, it is likely that this was due to the presence of an inhibitor of TAQ polymerase. These four cases were not included in further analyses or in Table 1.

To investigate the presence of mutations in genes of the ras family, the amplified DNAs were dotted onto nylon filters and hybridized to oligonucleotide probes. An example of such an experiment is shown in Fig. 1. Replica filters were hybridized to probes complementary to wild type and mutant sequences at N-ras codon 12/13. While most samples generated a strong signal on hybridization to wild type probe (Fig. 1i) only one case hybridized to a probe complementary to the N-ras codon 12 serine mutation (GGT→AGT) (Fig. 1ii) and a further case to a probe complementary to a codon 13 arginine mutation (GGT→CGT) (Fig. 1iii). Whenever a candidate ras mutation was detected on a dot blot, a fresh aliquot of tumor DNA was subjected to the PCR, run on a 2% (w/v) agarose gel and blotted onto a nylon filter by capillary transfer to confirm the result. We have found that this procedure often yields a lower background hybridization signal than direct application by dot blotting. Examples are illustrated in Figs. 2 and 3. In Fig. 2 replicate gel blots have been hybridized to N-ras codon 61 wild type (Fig. 2i) and lysine probes (CAA→AAA) (Fig. 2ii) while Fig. 3 shows replicate gel blots which have been hybridized to K-ras codon 12/13 wild type (Fig. 3i), codon 12 cysteine (GGT→TGT) (Fig. 3ii), and codon 13 aspartate (GAC→GAC) (Fig. 3iii).

Five ras gene mutations were found in this series of rhabdomyosarcoma (Table 1, Figs. 1, 2, and 3), three in N-ras, and two in K-ras. Examination of the clinical and pathological features of these cases reveals that all are rhabdomyosarcomas of embryonal type. Although there were no obvious correlations with the spectrum of histological appearances within this group, all five cases arose from the region of the genitourinary tract and none from the limbs or head and neck which constitute the other common sites of origin. In one case (Case 10, N-ras codon

Table 1 Rhabdomyosarcoma cases examined for ras gene mutations

<table>
<thead>
<tr>
<th>Case</th>
<th>Age and Sex</th>
<th>Site</th>
<th>Diagnosis</th>
<th>ras mutation</th>
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<tbody>
<tr>
<td>1</td>
<td>4F</td>
<td>Bladder</td>
<td>E*</td>
<td>K13Asp</td>
</tr>
<tr>
<td>2</td>
<td>2M</td>
<td>Calf</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3M</td>
<td>Calf (rec)</td>
<td>E</td>
<td>N61Lys</td>
</tr>
<tr>
<td>4</td>
<td>5M</td>
<td>Testis (rec)</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11F</td>
<td>Parotid</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>39F</td>
<td>Thigh</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3F</td>
<td>Tongue</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>19F</td>
<td>Retropertioneum</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>16M</td>
<td>Testis (rec)</td>
<td>E</td>
<td>N13Arg</td>
</tr>
<tr>
<td>10</td>
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<td>N13Arg</td>
</tr>
<tr>
<td>11</td>
<td>31M</td>
<td>Penis</td>
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<tr>
<td>12</td>
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<td>20M</td>
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<td>15</td>
<td>29M</td>
<td>Abdomen</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>18M</td>
<td>Chest (rec)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>12F</td>
<td>Perineum</td>
<td>A</td>
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<tr>
<td>18</td>
<td>43M</td>
<td>Thigh</td>
<td>P</td>
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<tr>
<td>19</td>
<td>62M</td>
<td>Thigh</td>
<td>P</td>
<td></td>
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<tr>
<td>20</td>
<td>57M</td>
<td>Axilla</td>
<td>P</td>
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<tr>
<td>21</td>
<td>41M</td>
<td>Testis</td>
<td>P</td>
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* E, embryonal; A, alveolar; P, pleomorphic; rec, recurrence; PAN, para aortic nodes.

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Rhabdomyosarcomas are tumors which show features of skeletal muscle differentiation. Three histological subtypes are recognized (21). Most are embryonal rhabdomyosarcomas, primitive neoplasms which usually arise in the first and early second decades of life and which possess many properties similar to other embryonal tumors such as neuroblastoma, medulloblastoma, and retinoblastoma. Alveolar rhabdomyosarcoma appears slightly later, usually during the second decade, and is characterized by the presence of broad bands of dense fibrous tissue which separate aggregates of tumor cells. Finally, the rare pleomorphic rhabdomyosarcoma is usually a tumor of adults containing large, bizarre cells. The majority of primary rhabdomyosarcomas arise from the head and neck region, the limbs and around the genitourinary tract. Although these sarcomas are highly malignant lesions and usually produce distant metastases, the prognosis has improved in recent years with the development of effective chemotherapeutic regimes.

We have examined a series of rhabdomyosarcomas for the presence of transforming mutations in H-, K-, and N-ras which might be involved in oncogenesis. The study shows that ras genes are mutated at the positions examined in approximately 35% (5/14) of embryonal rhabdomyosarcomas. This is therefore the first category of sarcoma or embryonal tumor identified in which activation of ras to transforming activity is a common event. The mutations in our series are in both N- and K-ras and are spread over many codons. This pattern differs from that found in colon carcinomas, pancreatic tumors and acute myeloid leukemia, where the reported activating mutations tend to be clustered at a single position of a particular ras gene (12, 15–18). It is, however, more reminiscent of the pattern described in thyroid neoplasms where mutations are spread over the H-, K-, and N-ras genes (24).

All the ras gene mutations detected in this report and in previous NIH 3T3 transfection studies (1, 22) were in embryonal rhabdomyosarcomas rather than alveolar or pleomorphic tumors. Since the latter two groups constitute a minority of cases both in the population and in our series, a more extensive study will be required to determine whether ras activation is really specific to the embryonal tumors. Nevertheless, this finding is reminiscent of observations on human lung carcinomas (25, 26) in which K-ras activation occurs frequently in adenocarcinomas but is rare in other non-small cell neoplasms. Within the group of embryonal rhabdomyosarcomas there appears to be no correlation between ras gene activation and histological features such as the degree of differentiation although we have noted that all our cases of ras mutation and in addition the previously reported cell line RD, are embryonal rhabdomyosarcomas arising from the genitourinary tract.

In the single case of recurrence of a tumor containing a mutated ras gene, the later specimen contained the same mutation as the earlier one. In the period between the two biopsies, the patient had been treated by chemotherapy. Farr et al. (17) have reported that in acute myeloid leukemia, recurrence after...
the disease has been put into remission by combination chemotherapy commonly lacks the ras gene mutation present in the earlier specimen. This has been attributed to the presence of multiple clones within the tumor, presumably with differing chemosensitivity. We shall also be carrying out studies on additional cases of recurrent rhabdomyosarcoma in order to investigate whether in some cases this is true for rhabdomyosarcoma. Finally, three of our cases of ras mutation (Cases 1, 5, and 12) and one of the cases previously studied by transfection (1) were in children under 5 years of age. These results therefore indicate that etiological factors implicated in the induction of ras gene point mutations may be active during early childhood.

Three other types of genetic change have previously been reported in human rhabdomyosarcoma; loss of DNA segments on chromosome 11 (27, 28), a consistent translocation between chromosomes 2 and 13 (29), and occasional amplification of myc genes (30–32). The relationship between different genetic events in rhabdomyosarcoma warrants further investigation. For example in colonic neoplasms, activating mutations in ras genes commonly coexist with loss of putative tumor suppressor elements on chromosomes 17 or 18 (33). Therefore in this neoplasm, the two forms of genetic alteration appear to represent independent and cooperative steps in tumorigenesis. A similar relationship between ras activation and loss of the putative tumor suppressor gene on chromosome 11 may exist in embryonal rhabdomyosarcomas. Conversely these changes could represent mutually exclusive pathways to oncogenesis. To clarify this point we are preparing a further study in which deletion of chromosomal segments on chromosome 11 will be investigated in parallel with ras gene mutation.

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