

## Frequency of Active *ras* Oncogenes in Human Bladder Cancers Associated with Schistosomiasis

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The frequency of active *ras* oncogenes in human bladder cancers associated with schistosomiasis, the cause of which is suspected to be a chemical carcinogen(s) in urine, was examined. Of 9 squamous cell carcinomas of the bladder surgically obtained in Egypt, none scored as positive in the regular DNA transfection assay using NIH/3T3 cells as recipients. The restriction fragment length polymorphism assay at codon 12 of the H-*ras* gene confirmed the absence of an activating mutation at this site in all of them. Western blotting analysis of electrophoretic mobilities of the *ras* p21 proteins, a method which can detect at least some of the point mutations within codons 12 and 61 of *ras* genes, suggested a point mutation within codon 61 in one out of the 7 tumors analyzed. In contrast to the low frequency of detection of mutationally activated *ras* oncogenes, enhanced expression of the *ras* p21 proteins was demonstrated in 4 of them by this analysis. The carcinogenic process involved in the endemic bilharzial bladder cancers is thus not associated with detectable point mutations within *ras* genes at a higher frequency than those in non-bilharzial bladder cancers in Japan or the USA.

Key words: Bladder cancer — *ras* oncogene — Schistosomiasis — p21 — DNA transfection

Transfection of NIH/3T3 cells with DNA has allowed the identification of oncogenes in a variety of naturally occurring human malignancies.<sup>1,2</sup> The oncogenes most frequently detected belong to three highly conserved members of the *ras* gene family, H-, K-, and N-*ras*, all of which encode closely related proteins generically designated *ras* p21.<sup>1</sup> Previously, we have screened a number of primary human tumors of the urinary tract obtained in Japan and the USA by the DNA transfection assay for such activated oncogenes; we found H-*ras* oncogenes and estimated the incidence at around 10%.<sup>3,4</sup> This figure contrasts with those found in animal tumors of some organs induced by chemical carcinogens; mutated H-*ras* oncogenes have been detected at a very high frequency, probably as a function of the specific carcinogen used and the tissue type.<sup>5,6</sup>

Bladder schistosomiasis (bilharziasis) caused by *Schistosoma haematobium* is endemic in the Nile valley, and there is a good

geographic coincidence between the incidence of bladder cancer and the intensity and prevalence of bilharziasis.<sup>7</sup> In Egypt, the incidence of bladder cancer is unusually high at around 20% of all cancers, superimposed on the bilharzial bladder symptoms, and found in younger patients compared with those without bilharzial infection.<sup>8</sup> Recently, Hicks *et al.*<sup>7,8</sup> found an association of elevated levels of urinary nitrosamines and bacteriuria with bilharziasis, and suggested that N-nitroso compounds could act as carcinogens for these bladder cancers. We therefore investigated in the present study whether the incidence of activated *ras* oncogenes in bilharzial bladder cancers, suspected to be caused by chemical carcinogens, is higher than that found in non-bilharzial bladder cancers and more nearly approaches that found in some chemically induced animal tumors.

### MATERIALS AND METHODS

**Tumors and Cells** Nine specimens of bladder carcinoma were obtained from nine patients, 7 males and 2 females, who underwent total cystectomy at

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the National Cancer Institute in Cairo. The carcinomas were all associated with schistosomiasis and were diagnosed pathologically as invasive squamous cell carcinomas of the bladder. Normal bladder mucosa was also obtained from 2 patients operated on for non-malignant lesions.

Mouse NIH/3T3 cells and NIH/3T3 cells transformed by the T24 bladder-cancer oncogene (T24 transfectant) were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. A human fibroblast cell line containing codon 61 activated *H-ras* gene (J. S. Rhim, unpublished data) and a normal human fibroblast cell line (IMR-90) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

**DNA Transfection** High-molecular-weight DNAs were extracted from tissues and cultured cells, and used to transfect NIH/3T3 cells by the calcium phosphate precipitation technique as previously described.<sup>3,4)</sup> Briefly, NIH/3T3 cells, seeded 24 hr earlier at  $1.3 \times 10^5$  cells per 10 cm dish, were exposed for 18 hr to the DNA precipitates (40  $\mu\text{g}$ /dish). Cultures were maintained with twice-weekly changes of medium and the number of transformed foci was counted at 28 days after transfection. Control assays using DNAs from the T24 transfectant (positive control) and sonicated human placental DNA (negative control) were included in each experiment, and only the foci morphologically different from those in the negative control dishes were counted.

**DNA Blotting Analysis** DNAs from primary tumors were digested with appropriate restriction endonucleases, electrophoresed on 1.0% or 1.8% agarose gels, and transferred to nitrocellulose filters. They were hybridized under stringent conditions with nick-translated <sup>32</sup>P-labeled DNA probes as previously described.<sup>4)</sup>

**Immunoprecipitation and Western Blotting of the *ras* p21 Proteins** Metabolic labeling of cells with [<sup>35</sup>S]methionine and immunoprecipitation of the *ras* p21 proteins using a monoclonal anti-p21 antibody (MAb Y13-259) were done as described by Srivastava *et al.*<sup>9)</sup> For Western blotting, lysates of frozen tissues containing 900  $\mu\text{g}$  of protein or of the T24 transfectant containing 100  $\mu\text{g}$  of protein were first immunoprecipitated with an excess of the MAb Y13-259.<sup>9)</sup> The resultant immune-complexes were resolved on 12.5% polyacrylamide slab gels containing SDS. Western blotting was performed as described by Papageorge *et al.*<sup>10)</sup> with slight modifications. After electrophoretic transfer of proteins from the gel onto nitrocellulose filters, p21 was detected by the use of a rabbit polyclonal anti-p21 antibody (kindly provided by S. K. Srivastava, NIH, Bethesda) and <sup>125</sup>I-labeled protein A. This polyclonal antibody as well as the MAb

Y13-259 reacts with p21 of H-, K-, and N-*ras* genes regardless of the presence or absence of activating point mutations within the coding sequences (S. K. Srivastava, personal communication).

Quantitation of differences in the levels of p21 was performed by densitometric analysis. Immunodetection of  $\beta_2$ -microglobulin was done by the same procedure except that lysates containing 15  $\mu\text{g}$  of protein were resolved on the gel without prior immunoprecipitation, and were detected by the use of anti human  $\beta_2$ -microglobulin (Miles Scientific, Illinois).

## RESULTS

**Absence of Transforming Genes Detectable by DNA Transfection Assay** Tumor DNAs from 9 patients were analyzed by DNA transfection assay using NIH/3T3 cells as recipients. Although DNA from the T24 transfectants induced an average of 0.52 foci per  $\mu\text{g}$  of DNA, no transformed foci was induced after transfection of 480  $\mu\text{g}$  of DNA per sample.

**Absence of Point Mutations within Codon 12 of *H-ras* Gene** All human *ras* oncogenes so far analyzed have been activated by single point mutations within codon 12, 13 or 61.<sup>2,11)</sup> Since an alteration at codon 12 of human *H-ras* gene leads to loss of a restriction site for *HpaII*/*MspI* digestion, thus providing a means of molecular genetic diagnosis of lesions at this position,<sup>4,12)</sup> the nine specimens of DNA were further surveyed for this restriction site polymorphism. When DNAs from the 9 tumors were digested with *MspI* and *HpaII* restriction endonucleases and separated on 1.9% agarose gels, all of them demonstrated a 355-base-pair (bp) band hybridizing with the *H-ras* specific pKY-1 probe,<sup>4)</sup> like that observed with human placenta, while DNA from the T24 transfectant containing codon 12-mutated human *H-ras* oncogene<sup>1)</sup> demonstrated the expected 411-bp band (data not shown). These results clearly indicated that the *H-ras* genes in these tumors do not have point mutations affecting the 12th amino acid of the encoded p21 protein.

**Altered Electrophoretic Mobility and Enhanced Expression of the *ras* p21 Proteins** Previously, Srivastava *et al.*<sup>9)</sup> reported that the characteristic alterations of either faster or slower electrophoretic mobility of the *ras* p21 proteins usually accompanied the activating lesions at codons 61 and 12, respectively. As

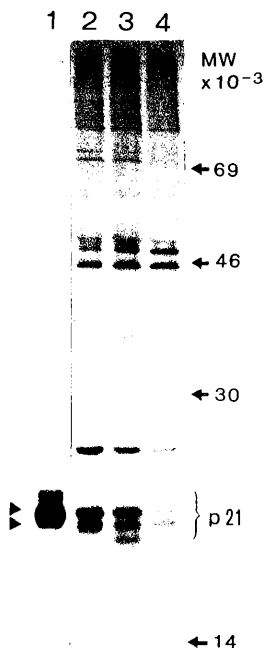


Fig. 1. Immunoprecipitation of the *ras* p21 proteins. T24 transfectants containing a codon 12-activated human H-*ras* gene (lane 1), NIH/3T3 cells (lane 2), human fibroblasts containing a codon 61-activated H-*ras* gene (lane 3), or IMR-90 human fibroblasts (lane 4) were labeled with [<sup>35</sup>S]-methionine. After immunoprecipitation with anti-p21 antibody (Y13-259), p21 electrophoretic mobilities were compared by 12.5% SDS/polyacrylamide gel electrophoresis and fluorography as described.<sup>9</sup> The p21 proteins expressed by control fibroblasts were used as a marker of normal p21 electrophoretic mobility and are indicated by arrowheads.

Fig. 1 shows, the normal p21 proteins of mouse (lane 2) and human (lane 4) origin appeared as a pair of bands under the present experimental conditions, the slower-moving band probably representing the p21 protein encoded by the normal K-*ras* gene<sup>6</sup> or the pro-p21 protein<sup>9</sup> or both. As expected, the

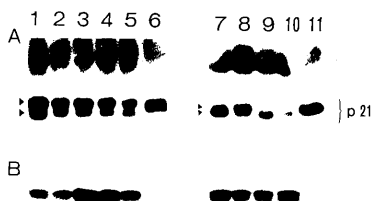


Fig. 2. Electrophoretic mobilities and expressions of *ras* protein p21. (A) Proteins extracted from tissues were immunoprecipitated with a monoclonal anti-p21 antibody (Y13-259), resolved on 12.5% SDS/polyacrylamide gel, Western-blotted and visualized by using a polyclonal anti-p21 antibody and [<sup>125</sup>I]protein A. (B) Amounts of  $\beta_2$ -microglobulin expressed were determined by resolving proteins without prior immunoprecipitation (15  $\mu$ g/sample) on the same gel as (A), and were visualized by using anti- $\beta_2$ -microglobulin and [<sup>125</sup>I]protein A. Extracts were from bilharzial bladder cancers EBT 21 to 24 (lanes 1 to 4, respectively), EBT 25 (lane 9), EBT 26 and 27 (lanes 7 and 8, respectively), normal bladder mucosa (lanes 5 and 10), and T24 transfectants (lanes 6 and 11). The amounts of protein applied to lanes 6 and 11 were one-ninth of those applied to other lanes. Lanes 1 to 6 and lanes 7 to 11 represent independent experiments.

T24 transfectants containing codon 12-mutated human H-*ras* oncogene (lane 1) and the human fibroblasts containing codon 61-mutated H-*ras* oncogene (lane 3) gave p21 proteins which moved slower or faster, respectively, than the normal p21 (indicated by arrowheads). We therefore assessed the presence of *ras* p21 proteins with such altered electrophoretic mobility in 7 of the 9 tumors analyzed above by combined use of immunoelectrophoresis and Western blotting. As shown in Fig. 2, the p21 proteins in normal bladder mucosa appeared either as a pair of bands (lane 5) or a fused single band (lane 10) in this analysis. In one of the 7 bladder tumors analyzed (EBT25, lane 9), the p21 was found to move faster than the normal p21. Although this sample scored as negative in the DNA transfection assay, it always gave a fast-moving p21 band in three independent experiments (data not shown), suggesting the

presence of a point mutation within codon 61 of a *ras* gene. However, the confirmation and identification of this probably mutated *ras* oncogene are still necessary, and the reason why the normally moving p21 bands were not obvious in this sample in addition to the fast-moving p21 band remains to be determined. The other 6 tumors yielded p21 proteins with normal mobility, while the T24 transfectant (lanes 6 and 11) yielded p21 with a slower-than-normal mobility, as expected. This result indicated that mutational activation of the *ras* oncogenes detectable by the present assay methods is not frequent, at most one in 7 tumors, in squamous cell carcinomas of the bladder associated with bilharziasis in Egypt. It should be noted, however, that 4 out of 7 tumors (lanes 1, 2, 7 and 8) expressed increased levels of the p21 proteins, when normalized with respect to the amount of  $\beta_2$ -microglobulin expressed (Fig. 2-B) and compared with those found in the normal bladder mucosa (lanes 5 and 10).

**Absence of *ras*-Gene Amplifications** Since increased levels of expression as well as a qualitative change in the *ras* gene have been associated with malignant transformation *in vitro*,<sup>13</sup> and amplified *ras* genes have been identified in bladder tumors,<sup>4,14</sup> we next assessed the presence of *ras*-gene amplification, one of the major mechanisms for in-

creased expression of p21, in these bilharzial bladder cancers. When tumor DNAs were digested with restriction endonuclease *Bam*HI or *Eco*RI, Southern-blotted and hybridized with a <sup>32</sup>P-labeled H-, K- or N-*ras* specific DNA probe, 9 of 9 tumor DNAs showed almost equal intensity of respective proto-oncogene fragments to those of normal human placenta DNA, indicating the absence of *ras*-gene amplification in these tumors (Fig. 3 for K-*ras* gene; other data not shown). Note the presence of extra bands in lanes 1 and 2 of Fig. 3; they reacted with a pBR322 probe (data not shown) and were probably due to the presence of microorganisms contaminating the primary tumor tissues.

## DISCUSSION

The H-*ras* oncogenes activated by somatic alterations within codon 61 have been identified in about 10% of primary tumors of the urinary tract by means of the regular DNA transfection assay using NIH/3T3 cells as recipients.<sup>3,4</sup> However, DNA transfection assay is a biological assay, and different samples of DNA may differ in their ability to transfect genes. Furthermore, there are greater than 1000-fold differences in transforming efficiency among members of the mutated *ras* genes,<sup>15</sup> so that some of the *ras* oncogenes might escape detection by the DNA transfection assay alone. Indeed, recent studies utilizing new molecular-biological methods have demonstrated the presence of K-*ras* genes with mutations at codon 12 in over one-third of primary human colorectal cancers.<sup>16,17</sup> Since the H-*ras* oncogene seems to be the most frequently activated oncogene in human bladder cancers,<sup>4</sup> we supplemented the present survey with the restriction endonuclease analysis that can detect all the activating mutations at codon 12 of the human H-*ras* gene, and with the analysis of the *ras* p21 proteins by Western blotting, which is especially sensitive in detecting codon 61-activated *ras* oncogenes (S. H. Reynolds, personal communication). Only with the last method could we detect one probable *ras* oncogene, although none was found by the other methods, out of 9 tumors. This indicated that the frequency of detectable *ras* oncogenes in bilharzial bladder cancers is not high and is almost the same as that in non-bilharzial bladder cancers ob-

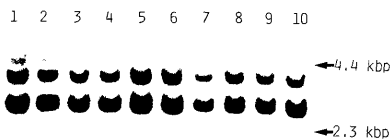


Fig. 3. Absence of amplified K-*ras* gene in bilharzial bladder cancers. DNAs (20  $\mu$ g) were digested with *Eco*RI and hybridized to a human K-*ras* 4th exon-specific probe.<sup>4</sup> The upper 3.6-kbp band corresponds to the K-*ras* pseudogene (K-*ras*-1) and the lower 2.4-kbp band corresponds to the K-*ras* protooncogene (K-*ras*-2).<sup>4</sup> Human DNAs were obtained from bilharzial bladder cancers EBT 21 to 29 (lanes 1 to 9, respectively) and human placenta (lane 10). Co-electrophoresed DNA fragments of *Hind*III-digested  $\lambda$ cI859 DNA served as molecular weight standards.

tained in Japan and the USA. The present finding that 4 out of 7 tumors analyzed by Western blotting using anti-p21 antibodies demonstrated increased levels of p21 compared with that found in normal bladder mucosa was also similar to the finding in non-bilharzial bladder cancers; by using an immunohistochemical technique, Viola *et al.*<sup>18)</sup> demonstrated that there were correlations between the reactivity with anti-p21 antibody and the pathological findings of the bladder tumors. However, the identity of the increased *ras* p21 and the underlying mechanisms of this increase (apparently not by gene amplification in the present case) remain to be determined.

In animal model systems, some of the chemical carcinogens have been shown to activate a member of the *ras* oncogene family, *H-ras*, by specific point mutations at very high frequencies.<sup>5,6)</sup> However, the carcinogen used and the incidence and kind of mutations caused were often correlated, and the carcinogens used in the previous studies were not nitrosamines, which have been suspected to be implicated in the case of bilharzial bladder cancers.<sup>7)</sup> Indeed, in a rat model system, oral administration of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine for 5 months frequently induced bladder tumors with enhanced expression of p21, but rarely induced tumors with mutationally activated *ras* oncogenes detectable by the NIH/3T3 transfection assay (J. Fujita *et al.*, unpublished data). Thus, the present finding that the frequency of detectable mutationally activated *ras* oncogene was not high in the bilharzial bladder cancers suggests that if chemical carcinogens are involved, they are not of the kind that frequently activates *ras* oncogenes by specific point mutations, or the mutations caused are not easily detectable by the present assay methods, which is consistent with the hypothesis that nitrosamines are involved in the pathogenesis of these cancers. By further investigating how and to what extent enhanced expression of p21 alters the regulatory controls of urothelial cell proliferation, we should achieve a better understanding of the carcinogenic process in the bilharzial bladder, and this might contribute to the management of patients.

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