Urol Res (1988) 16:415–418

Urological Research

Detection of *ras* Oncogenes by Analysis of p21 Proteins in Human Tumor Cell Lines

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Accepted: May 30, 1988

Summary. To detect mutationally activated *ras* oncogenes, we analyzed electrophoretic mobilities of *ras* p21 proteins utilizing the fact that many *ras* oncogenes produce abnormal p21 proteins that migrate at SDS/polyacrylamide gel electrophoresis as a fast-moving or slow-moving species in comparison to a normal p21 depending on the kind of mutation. Of 18 human tumor cell lines analyzed, four (SW480, SW620 and SW403 colon cancers, and SW626 ovary cancer) produced p21 belonging to the slow-moving species, suggesting a point mutation within codon 12 of a member of the three *ras* genes, H-, Ki- and N-*ras*. Subsequent DNA transfection analysis using NIH/3T3 cells as recipients identified activated Ki-*ras* oncogenes in the same four but not in other 14 cell lines. Thus, the analysis of p21 might serve as a rapid primary method to screen a large number of tumor materials for the presence of certain types of mutationally activated *ras* oncogenes.

Key words: Oncogenes – DNA transfection – *ras* p21 proteins

Introduction

The development of techniques for DNA transfection in eukaryotic cells has led to the discovery of cellular oncogenes in various human tumor cell lines and primary tumors [15]. The majority of oncogenes detected by their ability to transform NIH/3T3 cells in vitro have been shown to be three highly conserved members of the *ras* gene family, H-, Ki- and N-*ras*, all of which encode closely related proteins generically designated p21. Normal cellular p21 can acquire transforming activity by structural alterations, which so far have been a consequence of single point mutations within codons 12, 13 or 61 of their coding sequences in the human tumors [1, 15].

Some evidence indicates that 5–20% of human tumors contain mutationally activated *ras* oncogenes detectable by the DNA transfection assay [15]. We have surveyed 38 primary tumors of the urinary tract by the DNA transfection and found activated H-*ras* oncogenes in two cases [9]. Since the number of positive cases was so small, we could not draw any conclusions about the relationship between the presence of active *ras* oncogene and clinico-pathological findings of the patient. The use of DNA transfection in a large scale screening for active oncogenes, however, has several limitations; e.g. it is very laborious and requires large volumes of tissues for DNA preparation.

Previously, Scrivastava et al. [13] have studied the effects of the two major activating lesions on the electrophoretic mobility of p21 *ras* proteins, and found that the characteristic alterations of either faster of slower mobility at SDS/polyacrylamide gel electrophoresis (SDS/PAGE) appeared to accompany the activating lesions at codons 12 and 61, respectively. Therefore, we examined in the present study whether the analysis of p21 could be a primary screening method to detect mutationally activated *ras* oncogenes. We applied this method to 18 human tumor cell lines and compared the result obtained with that of the DNA transfection assay.

Materials and Methods

Cells

Eighteen human tumor cell lines were screened for the presence of active *ras* oncogenes (Table 1). T24 bladder cancer cell line and Hs242 lung cancer cell line, and IMR-90 human fibroblast cell line were used as oncogene-containing controls and a normal control, respectively [15].

Analysis of Electrophoretic Mobilities of p21 Proteins

Metabolic labeling of cells and immunoprecipitation of the *ras* p21 proteins were done as described by Srivastava et al. [13]. Briefly, [^{35}S]-methionine labeled cells were extracted with 1% Triron X-100,

Table 1. 18 human tumor cell lines analyzed

Organ	Cell lines
Kidney	SK-NEP-1[a], CAKI-1[a], CAKI-2, A-704
Prostate	DU-145[a], PC-3[a]
Testis	TERA-1[a], TERA-2[a], CATES-1B[a]
Ovary	SW626, CAOV-3, CAOV-4
Colon	SW480[a], SW620[a], SW403, LS-180, DLD-1, HCT-15

[a] Cell lines known to be derived from primary tumors with metastasis or metastatic tumors

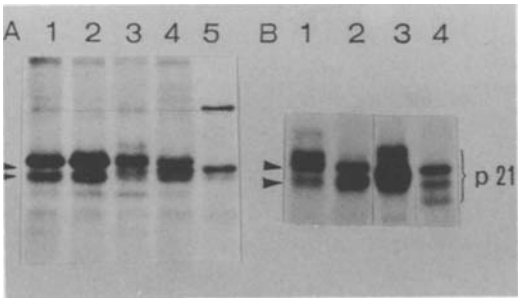

Fig. 1A and B. Electrophoretic mobilities of p21 *ras* proteins in human tumor cell lines. **A** [^{35}P]-methionine-labeled cell extracts were immunoprecipitated with an anti-p21 monoclonal antibody (Y13-259), and analyzed by electrophoresis on 12.5% SDS/polyacrylamide gels. Lane 1, SW480 colon cancer; lane 2, SW620 colon cancer; lane 3, SW403 colon cancer; lane 4 SW626 ovary cancer; lane 5, IMR-90 normal fibroblast. **B** Proteins extracted from cultured cells were immunoprecipitated with Y13-259, resolved on a gel, Western blotted, and visualized using a polyclonal anti-p21 antibody and [^{125}I]-protein A. Lane 1, SW626 ovary cancer; lane 2, IMR-90 fibroblast, lane 3, T24 bladder cancer; lane 4, Hs242 lung cancer. Arrowheads indicate the positions of normal p21 bands

Table 2. Detection of oncogenes by DNA transfection

Cell line	Number of foci/μg of DNA
SW480 colon cancer	8/320
SW620 colon cancer	7/320
SW403 colon cancer	4/320
SW626 ovary cancer	4/320
Other tumors (14 cell lines)	0/320 (each)
IMR-90 fibroblast	0/960

0.1% SDS, 0.5% sodium deoxycholate, 0.1 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM phenylmethanesulfonyl fluoride, and aprotinin (100 Kallikrein inactivator units/ml). The lysate containing 4 x 10^7 trichloroacetic acid-precipitable counts per minute was incubated with a monoclonal anti-p21 antibody (MAb Y13-259), and then precipitated with protein-A-Sepharose coated with goat anti-rat immunoglobulin G. SDS/PAGE of immunoprecipitated samples was done in 12.5% polyacrylamide slab gels as described previously [7, 8].

For Western blotting, lysates containing 1,000 μg of protein from unlabeled cells were first immunoprecipitated with MAb Y13-259. They were then resolved on SDS/polyacrylamide gels and blotted onto nitrocellulose filters. The p21 bands were visualized using a polyclonal anti-p21 antibody and [^{125}I]-labeled protein A as described previously [7, 8]. This polyclonal antibody as well as MAb Y13-259 has demonstrated reactivity with both the point-mutated and proto-onc forms of *ras* p21 encoded by all 3 members of the *ras* gene family.

DNA Transfection Assay

High molecular weight DNAs extracted from the cultured cells were transfected onto NIH/3T3 cells by the calcium phosphate precipitation technique as described [9]. Briefly, NIH/3T3 cells, seeded 24 h earlier at 1.3 x 10^5 cells per 10 cm dish were exposed for 18 h to the DNA precipitates (40 μg/dish). The number of transformed foci were counted at 28 days after transfection. Control assays using DNA from T24 transfectant (positive control) and sonicated human placental DNA (negative control) were included in each experiment, and only those foci morphologically different from those in the negative control dishes were counted.

DNA Blotting Analysis

DNA from the primary transfectants were digested with appropriate restriction endonucleases, electrophoresed on 1.0% agarose gels, and transferred to nitrocellulose filters. They were hybridized with nick-translated [^{32}P]-labeled DNA probes under stringent conditions as described previously [10].

Results

Altered electrophoretic mobilities were classified as slow- or fast-moving species in comparison to normal human or mouse p21 bands (8, 13). The former suggests a point mutation within codon 12 and the latter suggests one within codon 61 of *ras* genes. Of 18 human tumor cell lines screened for p21 with such altered electrophoretic mobilities by metabolic labeling and p21 immunoprecipitation, 4 scored as positive (Fig. 1A). SW480 colon cancer cells (lane 1) yielded p21 that migrated slower than the normal human p21 bands (lane 5). Three other tumor cell lines, SW620 (lane 2), SW403 (lane 3), and SW626 (lane 4), also showed slow-moving p21, suggesting that they have lesions affecting the 12th codon of one of the *ras* genes.

The altered electrophoretic mobilities of p21 was also detected in non-labeled cells by Western blotting (Fig. 1B). A slow-moving p21 species was detected in SW626 cells (lane 1), and as expected, T24 with codon 12-activated H-*ras* gene (lane 3) and Hs242 with codon 61-activated H-*ras* gene (lane 4) [15] showed slow- and fast-moving p21 species, respectively.

As shown in Table 2, DNAs from SW480, SW620, SW403, and SW626 induced transformed foci in the DNA transfection assay. Since *ras* oncogenes have been shown to be most frequently detected by the DNA transfection assay [9, 15], we subjected individual transfectant DNA to Southern blotting analysis using probes specific to each member of the *ras* gene family. Neither H-*ras* nor N-*ras* specific probes

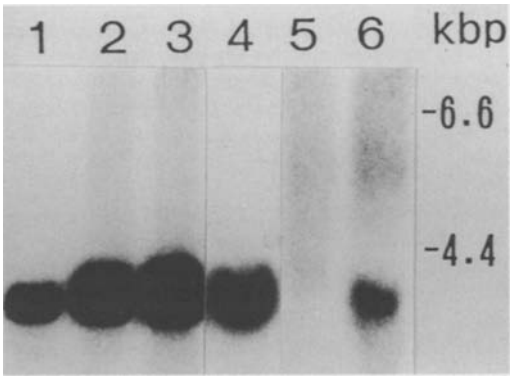

Fig. 2. Detection of sequences related to the Ki-*ras* oncogene in NIH/3T3 transfectants derived from the human tumor cell lines. High molecular weight DNA (20 μg) was digested with *Eco*RI and electrophoresed on 1% agarose gel, blotted onto a nitrocellulose filter and hybridized with [^{32}P]-labeled human Ki-*ras* specific probe. Cellular DNA was extracted from representative NIH/3T3 transfectants from SW480 cells (lane 1), SW620 cells (lane 2), SW402 cells (lane 3) and SW626 cells (lane 4) as well as NIH/3T3 cells (lane 5) and IMR-90 cells (lane 6)

detected any bands other than the endogenous mouse bands (data not shown). However, when EcoRI-digested transfectant DNA was probed with human Ki-*ras*-specific DNA, each transfectant DNA showed strong hybridizing bands not observed in mouse DNA (Fig. 2). These findings indicated that Ki-*ras* oncogenes were activated in these 4 tumor cell lines. In accord with the initial p21 analysis of the parental cell lines, each NIH/3T3 transfectant derived from SW480, SW620, SW403 and SW626 produced a p21 that migrated as a slow-moving species, suggesting a point mutation within codon 12 of the respective *ras* oncogenes transfected (data not shown). In summary of 18 human tumor cell lines surveyed for mutationally activated *ras* oncogenes by analysis of p21 and DNA transfection, 4 cell lines scored as positive in both assays.

Discussion

DNA transfection has identified the presence of mutationally activated *ras* oncogones in 5–20% of human malignancies [1, 9, 15] and at even higher fequency in some chemically induced or spontaneous animal tumors [18]. The importance of *ras* oncogenes in human malignancy has further been suggested by their specific activation in tumor tissues by somatic alterations [10], and by their ability to induce neoplastic conversion of human cells in vitro [12]. Although several oncogenes other than *ras* genes have been isolated from human tumors by the DNA transfection assay using NIH/3T3 cells as recipients [5], most of the oncogenes detected belong to the *ras* gene family [15]. In the present study, we detected oncogenes in 4 (SW480, SW620, SW403 and SW626) of 18 human tumor cell lines by the DNA transfection assay. These oncogenes were all identified as the activated Ki-*ras* oncogenes. Although SW626 was derived from the metastatic tumor the primary tumor of which gave the SW480 cell line, oncogene was not detected in other examined cell lines derived from metastatic tumors.

Recently human tumors have been screened for overexpression of *ras* genes, which is also shown to transform NIH/3T3 cells in vitro [15], using either histochemical or Western blotting, and correlation between expression of *ras* genes and clinico-pathological findings has been suggested in some cases [11, 16]. As for the mutationally activated *ras* oncogenes, correlation between the presence of a specific mutation of *ras* gene and clinico-pathological finding of the tumor has not been revealed, although the presence of an activated *ras* oncogene is shown to enhance a metastatic phenotype of mouse cancer cells [17]. One possible reason for this is the small number of activated oncogenes identified. Although the DNA transfection assay has several advantages, e.g., transfectants are stable and can be kept for further characterization, this takes more than 3 weeks to perform, thus making analysis of a large number of tumors difficult. Furthermore, since there are more than 1000-fold differences in transforming efficiency among members of mutated *ras* genes, some of them might escape detection by the DNA transfection assay [4]. For this reason, we evaluated the analysis of the electrophoretic mobilities of p21 *ras* protein as a primary screening method to detect mutationally activated *ras* oncogenes. It is less time consuming and requires a smaller amount of tissue and less technical skill compared with other methods [2, 6]. The present study suggested that it is as sensitive as the DNA transfection assay in detecting certain types of *ras* oncogenes even when applied to non-labeled tumor tissues. The presence of *ras* oncogene was detected in 4 of 18 human tumor cell lines by the analysis of p21, which was in complete accordance with the result of DNA transfection assay. It could also suggest the site of mutated codon within such genes. The slower than normal mobilities of p21 demonstrated in the four specimens suggested that 12th codon alterations were the activating lesions in each case. This was indeed the case at least for SW480 and SW620 tumor cells as demonstrated by others [3, 14, 15]. Furthermore, with appropriate controls, the analysis of p21 on the gel indicates the amount of p21 expressed [7, 11]. However, not all the possible activating lesions cause altered electrophoretic mobility of p21 [4]. Further studies using different amino acid substitutions at codons 12, 13 or 61 of each members of *ras* gene family should therefore be performed to define the usefulness and limitation of this approach.

The present study strengthens the notion that *ras* oncogenes activated by point mutations within codon 12 or 61 are most frequently detected in human tumors, and suggest that the analysis of p21 would be a practical screening method to detect at least certain subset of activated *ras* oncogenes. By accumulating a large number of clinical data, the significance of finding activated *ras* oncogenes in human cancers would become apparent and might contribute to the management of the patients.

Acknowledgements. This work was partly supported by Grants-in-Aid from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare, Osaka Anti-cancer Campaign, Osaka Cancer Research Fund and the Osaka Kidney Foundation (OKF88-0005).

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