IN SITU DETECTION OF HA-RAS AND C-MYC mRNA IN CANCER CELL LINES AND TISSUE SECTIONS OF COLORECTAL CANCER USING SULFONATED DNA PROBES

TAKUSHI MONDEN, HIDEKI MORIMOTO, MASAHICO HIGASHIYAMA,
MASAHIRO MUROTANI, NAOHIRO TOMITA, TAKASHI SHIMANO,
HIROSHI OKUDA AND TAKESADA MORI

*Department of Surgery II, Osaka University Medical School, Fukushima 1-1-50,
Fukushima-ku, Osaka 553*

We have previously reported the technique of *in situ* hybridization utilizing sulfonated DNA probes for the detection of amylase mRNA in formalin-fixed paraffin sections of human pancreas and submaxillary gland (22). Using this technique, we detected the mRNAs of Ha-ras and c-myc oncogenes in colonic cancer tissues from 24 patients, as well as in cultured cell lines. HL 60 cells, which are known to have amplified c-myc gene and accelerated transcription rate of this gene (11), and transformed NIH/3T3 cells with T24 Ha-ras were hybridized *in situ* with the sulfonated DNA probes of v-myc and v-Ha-ras respectively. Specific hybridization of v-myc probe for HL 60 and of v-Ha-ras probe for NIH/3T3 cells were clearly demonstrated at the cytoplasm by immunohistochemistry using a monoclonal antibody raised against sulfonated DNA. Of the colonic cancer tissues, which were freshly prepared and processed in paraffin, 6 were positive for Ha-ras mRNA and 7 were positive for c-myc mRNA. Northern blot analysis of the positive cases confirmed the validity of the results of *in situ* hybridization.

Several reports have demonstrated the amplification (3, 20, 21) or increased expression of ras and/or myc oncogenes in colorectal cancer. Over expression of c-Ha-ras and/or c-myc oncogenes has been confirmed by Northern blot analysis (2, 7, 23, 24) or immunological techniques using antibodies raised against oncogene products (8, 10, 15-18, 24, 28, 29, 32).

Northern blot or immunoblot analysis, however, represents the average of heterogeneous cell populations including many normal mesenchymal cells as well as cancer cells, and it is not possible to elucidate the increased expression of a reference oncogene in individual cells.

Thus, the immunohistological detection of oncogene products seems to provide much more valuable information especially for pathological researchers. Recently, several monoclonal antibodies have been raised against synthetic peptides encoded by Ha-ras or c-myc oncogene (14, 16, 26, 27). The results of immunohistological studies from independent laboratories using such monoclonal antibodies, however, have not always been consistent, and different patterns of oncogene expression have been described (8, 15-18, 24, 29, 32). The reason for the disagreement is obscure, but monoclonal antibodies raised against a very small part of antigen molecule may recognize the unrelated proteins having a similar tertiary structure.

The mRNA-DNA *in situ* hybridization technique, which makes it possible to

detect the gene transcripts in individual cells, is expected to provide a more direct proof of activation of the reference oncogene. In this experiment, we intended to detect Ha-ras and c-myc oncogene mRNAs in paraffin embedded tissue sections of colorectal cancer as well as in cultured cell lines using sulfonated DNA probes.

MATERIALS AND METHODS

Tissue samples

Small tissue samples obtained in endoscopic or colonoscopic examination of colorectal cancer were fixed immediately in 10% cold buffered formalin saline (4°C, pH 7.4) for 2–3 hr. The tissues were rinsed with distilled water several times, dehydrated through graded ethanol, infiltrated and embedded in paraffin. Four micron thick sections were made on a poly-L-lysinecoated glass slide and dried at 40°C for 2 hr. The sections were then dewaxed in xylene, washed in absolute ethanol, air-dried and stored at –20°C.

For Northern blot analysis, 1 cm³ of tumor specimens and normal colon mucosae remote from the edge of cancer were collected from surgical specimens. The tissue specimens were immediately frozen and stored in liquid nitrogen until analyzed.

Cell preparation

NIH/3T3 cells were transfected with activated c-Ha-ras gene of T24 bladder carcinoma (pT22 clone) in our laboratory. The transformants were cultured on glass slides for 24 hr in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum (DMEM-10% FCS), and the adherent cells were fixed in 10% cold buffered formalin saline (4°C, pH 7.4) for 5 min. After dehydration, the samples were stored at –20°C until used. A negative control of the untransformed NIH/3T3 cells was prepared in the same way.

A human promyelocytic leukemia cell line HL60 was obtained from the Japanese Cancer Research Resources Bank (JCRB; Tokyo, Japan) and cultured in DMEM-10% FCS. About 1×10^8 cells were centrifuged, pelleted, and fixed in 10% cold buffered formalin saline for 10 min. Paraffin sections of HL60 cells were prepared as described above.

Preparation and sulfonation of the probes

A 0.88 kb Hind III fragment of Ha-MSV (13) (Takara Shuzo, Japan) and a 0.99 kb Sal I-Pst I fragment of MC29 (1) (v-myc; Takara Shuzo, Japan) were used as hybridization probes for *in situ* hybridization. A 0.95 kb Sau 3A I fragment of pUC 19 vector DNA was also prepared as a control probe. Sulfonation of DNA probes was carried out by treating denatured DNA with a mixture of bisulfite and o-methylhydroxylamine as described elsewhere (6).

In situ hybridization

The method for *in situ* hybridization was described previously (22). Briefly, sections were rehydrated, treated with 0.2 N HCl, heated to 65°C in $2 \times$ SSC, and digested with 5 µg/ml proteinase K. After prehybridization for 4 hr, hybridization was carried out at 42°C for 12–18 hr using sulfonated v-Ha-ras, v-myc or pUC 19 probes. After extensive washing in $2 \times$ SSC at 37°C for several hours, specific hybridization signals were visualized immunohistochemically by the avidin-biotin peroxidase complex (ABC) method using a monoclonal antibody specific to sulfonated DNA (Organics, Israel).

Northern blot hybridization

Total cellular RNA was isolated from the tissues of colorectal cancer and normal colon mucosa essentially as described previously (9). The poly-A(+) RNA was purified from total RNA by repeated passages through an oligo dT cellulose column. An aliquot (5 µg) of mRNA was electrophoresed in 1% agarose/2.2 M formaldehyde gel (19) and then transferred to a nylon filter (Gene Screen Plus, NEN, USA.). After hybridization with ³²P-labeled v-Ha-ras or v-myc probe, the filter was exposed to X-ray film (Kodak XA-5) overnight at -70°C.

RESULTS

When NIH/3T3 cells transfected with T24 Ha-ras were hybridized *in situ* with the sulfonated v-Ha-ras, the cytoplasm of almost all the cells were stained diffusely (Fig. 1a). The control study using the NIH/3T3 without transfection showed only weak staining (Fig. 1b), and hybridization with unrelated sulfonated probe pUC gave no visible staining. The Southern blot analysis of the T24 Ha-ras transfected NIH/3T3 cells showed that the DNA of the transfected cells contained more than 6 copies of Ha-ras gene (data not shown). HL 60 cells, which are known to have a markedly amplified c-myc oncogene and accelerated transcription of this gene (11), were proved to have an accumulation of c-myc mRNA in the cytoplasm by sulfonated v-myc probe. In the case of HL 60, however, the staining pattern was heterogeneous, and some cells were not stained (Fig. 2).

Of 24 colorectal cancer tissue specimens, 6 stained positive for Ha-ras mRNA and 7 were positive for c-myc mRNA (Figs. 3a, b, 4). The cancer tissues examined were well or moderately differentiated, and one mucinous adenocarcinoma was included. In these cases, heterogeneity of staining intensity in the same section was observed by *in situ* hybridization (Fig. 3a).

Ten of the 24 colorectal cancer specimens were also examined by Northern blot

TABLE 1. Results of *in situ* hybridization and Northern blot analysis of 10 cases of colorectal cancer

Case No.	Age	Histol.	Ha-ras mRNA		c-myc mRNA	
			ISH	NB	ISH	NB
1.	53	Well	—	—	+	+
2.	60	Well	—	—	+	+
3.	80	Well	+	+	—	—
4.	50	Muc	+	—	+	+
5.	37	Mod	—	—	—	—
6.	58	Well	—	+	—	—
7.	61	Mod	—	—	—	+
8.	41	Well	+	+	+	N.T.
9.	57	Well	—	+	N.T.	N.T.
10.	75	Well	+	+	—	N.T.

ISH: *in situ* hybridization, NB: Northern blot analysis

N.T.: not tested.

FIG. 1. *In situ* hybridization of sulfonated v-Ha-ras (880 bp) to the NIH/3T3 cells with or without transfection of T24 c-Ha-ras gene. The transfectant cells show diffuse staining of cytoplasm (a), but the cells without transfection give only weak staining (b). Counterstained with methylgreen.

FIG. 2. *In situ* hybridization of sulfonated v-myc (990 bp) to the paraffin section of cultured HL 60 cells with hematoxylin counter staining. About half the number of HL 60 cells show diffuse cytoplasmic stainings, but the remainders give no visible staining. The diversity of staining pattern from cell to cell implicated the heterogeneity of the individual cell differentiation.

hybridization. Fig. 5 shows the result of Northern blot hybridization for Ha-ras mRNA from 5 of 10 specimens. Increase of oncogene mRNA extracted from the tumor was confirmed by comparison with that from normal mucosa remote from the tumor. Table 1. summarizes the results of *in situ* hybridization and Northern blot analysis of colorectal cancer.

DISCUSSION

By the *in situ* hybridization technique using sulfonated DNA probes, we examined Ha-ras and c-myc oncogene transcription in 2 cell lines and colorectal cancer tissues. NIH/3T3 cells of primary transfectants with T24 Ha-ras gene gave an intense cytoplasmic staining for v-Ha-ras probe. The staining of the individual cells was uniform and all cells showed hybridization by v-Ha-ras probe. Human promyelocytic leukemia cells, HL 60, however, were highly heterogeneous and hybridization by v-myc probe gave a wide range of staining intensities. It has been reported that HL 60 cells are capable of differentiation into granulocytic or monocytic forms by exposure of the cells in culture to various physiological and pharmacological agents (4, 129), and the activation and reduction of several oncogenes can occur according

FIG. 3. *In situ* detection of Ha-ras mRNA of colorectal cancer tissue using sulfonated v-Ha-ras probe. A low power view shows the heterogeneous staining in the section (a), and the specific hybridization is observed at the cytoplasm of cancer cells by high power magnification (b). Counterstained with hematoxylin.

FIG. 4. *In situ* detection of c-myc mRNA of colorectal cancer. The sulfonated v-myc probe hybridized to cancer cells is clearly demonstrated at the cytoplasm. Counterstained with hematoxylin.

Northern Blot Analysis (Ha-ras)

FIG. 5. Northern blots of c-Ha-ras mRNA from colon carcinoma samples (Lane 1–5) and normal colon mucosa (Lane 6). Samples (5 μ g) of polyadenylated RNAs from tumor tissues and normal mucosa were electrophoresed in 1% agarose gel and transferred to nitrocellulose, and hybridized by 32 P-labeled v-Ha-ras probe. Lane 2, 3 and 4 give a strong single band at 1.4 kb, and a weak band is observed in lane 1 and 5 as well as in the control lane 6. Lane 1 corresponds to case No. 4, lane 2 to No. 6, lane 3 to No. 3, lane 4 to No. 8, and lane 5 to No. 7 of Table 1 respectively.

to the cell differentiation (5, 25, 31). The results of our *in situ* hybridization of HL 60 cells by v-myc probe reflect the potential of differentiation of HL 60 cells in culture.

A survey of colorectal cancer tissue specimens from 24 patients by *in situ* hybridization disclosed accelerated transcription of Ha-ras gene in 6 and c-myc in 7. The results of *in situ* hybridization and those by Northern blot analysis agreed in most cases (see Table 1), but in 3 patients (Table 1, Nos. 6 and 9, Ha-ras; and No. 7, c-myc), Northern blot analysis gave positive band for Ha-ras or c-myc mRNA while *in situ* hybridization could not identify the positive cells with accelerated transcription of Ha-ras or c-myc gene. The reason for this discrepancy might be the fragility of the

mRNAs in the tissue blocks, which contained a large amount of RNase which might have digested considerable amounts of RNA in the course of routine processing into paraffin sections (30). Although we took every precaution to minimize the RNase activity by fixing the materials as soon as possible after biopsy and dehydrating them at low temperature, it may be impossible to entirely prevent the RNA from being broken into small fragments.

Another discrepancy between the *in situ* hybridization and the Northern blot analysis was observed in the specimen from patient No. 4, which was positive for Ha-ras mRNA by *in situ* hybridization but was negative by Northern blot analysis. In this case, the tumor cells produced copious mucin and the tumor was diagnosed as "mucinous adenocarcinoma" by histology. The sample from such mucinous carcinoma should contain a relatively small number of cancer cells compared to that of normal mesenchymal cells, which might cause the negative band of Ha-ras mRNA by Northern blot analysis.

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