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Technical Report Series

Expression of Proto-oncogenes in Non-Hodgkin's Lymphomas by In Situ Hybridization with Biotinylated DNA Probes

Kiyohiro Hamatani, Ph.D.; Kuniko Yoshida, M.D.; Masumi Abe, M.S.; Hisayoshi Kondo, Ph.D.; Katsutaro Shimaoka, M.D.; Hiroshi Shiku, M.D.; Mitoshi Akiyama, M.D.

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A Cooperative Japan–United States Research Organization

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The Radiation Effects Research Foundation (formerly ABCC) was established in April 1975 as a private nonprofit Japanese Foundation, supported equally by the Government of Japan through the Ministry of Health and Welfare, and the Government of the United States through the National Academy of Sciences under contract with the Department of Energy.

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業　績　報　告　書

RERF TR 6-89
研究課題　4-88

ビオチン化DNAプローブを用いた in situ ハイブリダイゼーションによる 非ホジキンリンパ腫における癌遺伝子の発現§

Expression of Proto-oncogenes in Non-Hodgkin's Lymphomas by In Situ Hybridization with Biotinylated DNA Probes

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要　約

43例の非ホジキンリンパ腫における6種類の癌遺伝子（fos，myc，myb，Ki-ras Ha-ras及びN-ras）発現をin situハイブリダイゼーション法により解析した．ビオチン化DNAプローブとして，6種類の癌遺伝子，イムノグロブリンH鎖（I_gH）遺伝子及びT細胞レセプターβ鎖（TCRβ）遺伝子を用いた．I_gH遺伝子及びTCRβ鎖遺伝子をプローブとして行ったin situハイブリダイゼーションの結果は，細胞表面マーカーにより分類された細胞型と一致した．核蛋白に関連している癌遺伝子，fos，myc及びmybは細胞型，組織病理学的分類あるいは悪性度に関係なく全症例の70%～80%において発現されていた．一方，悪性度の高いT細胞型の症例においてKi-ras遺伝子の発現が高頻度に見られる以外，rasファミリー遺伝子はより少数の症例にしか発現が認められなかった．数種類の症例から抽出したRNAを用いて行ったドットハイブリダイゼーションの結果はin situハイブリダイゼーションの結果と一致しており，このこともin situハイブリダイゼーションの反応が特異的であることを示している．

§要約以外の訳文はない

*放射線生物学部非常勤研究員及び長崎大学医学部附属病院検査部．**長崎大学医学部附属原爆被災学術資料センター．†放影研顧問及び長崎大学医学部腫瘍医学教室．

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Summary

Expression of six proto-oncogenes (*fos, myc, myb,* Ki-*ras,* Ha-*ras,* and N-*ras)* in 43 cases of non-Hodgkin's lymphoma was analyzed by means of in situ hybridization. Biotinylated DNA probes of the six oncogenes and those of the immunoglobulin H-chain (*IgH)* gene and the T cell receptor β-chain (*TCR*β) gene were used. The results of in situ hybridization performed under blind conditions by *IgH* and *TCR*β gene probes were compatible with those of typing by cell surface markers. The nuclear protein-related proto-oncogenes, *fos, myc,* and *myb,* were expressed in about 70%–80% of all cases regardless of phenotypes, histology or histologic grade. On the contrary, genes of the *ras* family were expressed in fewer cases except for the Ki-*ras* gene which was more frequently expressed by cases of the T cell immunophenotype with a high malignancy grade. The results of dot hybridization with RNA extracted from some cases were compatible with those of in situ hybridization, further demonstrating the specificity of in situ hybridization.

§ **The complete text of this report will not be available in Japanese.**

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Introduction

In situ hybridization for detecting mRNA using DNA or RNA probes is commonly used in the direct analysis of gene expression at the cellular or tissue level.[1–8] Rapidly increasing numbers of isolated genes make in situ hybridization an extremely useful analytical technique, since isolation and characterization of genes often precede molecular identification of the gene products. In addition, standard immunohistochemical analysis requires the production of antibodies with well-defined specificity, and such reagents are not always readily available. Despite its potential applicability to a wide variety of scientific fields, in situ hybridization still possesses various technical problems in terms of specificity and reproducibility of obtained results, complexity of procedures, and sensitivity of assays.

To establish an in situ hybridization assay for this study, non-Hodgkin's lymphomas (NHL) were used because all of the NHL analyzed were immunologically pretyped with cell surface markers, and also because *IgH* and *TCRβ* genes as probes of internal control were available for examining the specificity of in situ hybridization. Recently, an in situ hybridization assay using photobiotinylated DNA probes was developed in this laboratory with reliable specificity and reproducibility. Data resulting from this method can be summarized as follows: 43 cases of NHL were analyzed under blind conditions. The results of *TCRβ* and *IgH* gene expression were very compatible with those of immunological typing, thus demonstrating the specificity of this in situ hybridization technique. All *fos, myc,* and *myb* genes were uniformly expressed in about 80%–90% of B cell-type NHL, and in 59%–77% of T cell-type NHL. In contrast to this, all Ki-*ras,* Ha-*ras,* and N-*ras* were expressed in a more limited number of NHL cases. The results of dot hybridization with RNA extracted from selected cases correlated well with those of in situ hybridization—further evidence of the specificity of this in situ hybridization technique.

Materials and Methods

Lymphoma tissues

Biopsied lymphoma tissues were maintained at −80°C until use. Parts of specimens were used for histopathological diagnosis and also for typing of cell surface markers with monoclonal antibodies. Histopathological classification of NHL was performed according to the International Working Formulation.[9]

Typing of cell surface markers

Single-cell suspension was prepared from parts of biopsied samples, and expression of cell surface markers was analyzed either by the standard indirect immunofluorescence test or by fluorocytometry. As T cell markers, expression of CD 2, 3, 4, 5, and 8 was analyzed, whereas expression of B1, B4, and surface Ig was analyzed as B cell markers.

DNA probes

Prepared from plasmids, all DNA fragments shown in Figure 1 were used as probes for in situ hybridization. (*TCRβ* and c-*myc* genes were kindly provided by Dr. T. Naoe, Nagoya University, Nagoya. The *JH* segment of the *IgH* gene was a generous gift of Dr. M. Abe, RERF, Nagasaki.) Probes of v-Ki-*ras,* v-Ha-*ras,* and N-*ras* oncogenes were prepared from plasmids pHiHi-3,[10] pH-1,[10] and p6al,[11] respectively. Both v-*fos* and v-*myb* were prepared from p-*fos*-1[12] and p-*myb,*[13] respectively. (These oncogenes were kindly provided by the Japanese Cancer Research Resources Bank, Tokyo.) All inserts were purified by agarose gel or polyacrylamide gel electrophoresis and electroelution before photobiotinylation. Hind III restriction fragments of *JH* were digested with Sma I, a mixture of Hind III–Sma I restriction fragments being used for in situ hybridization (see Figure 1).

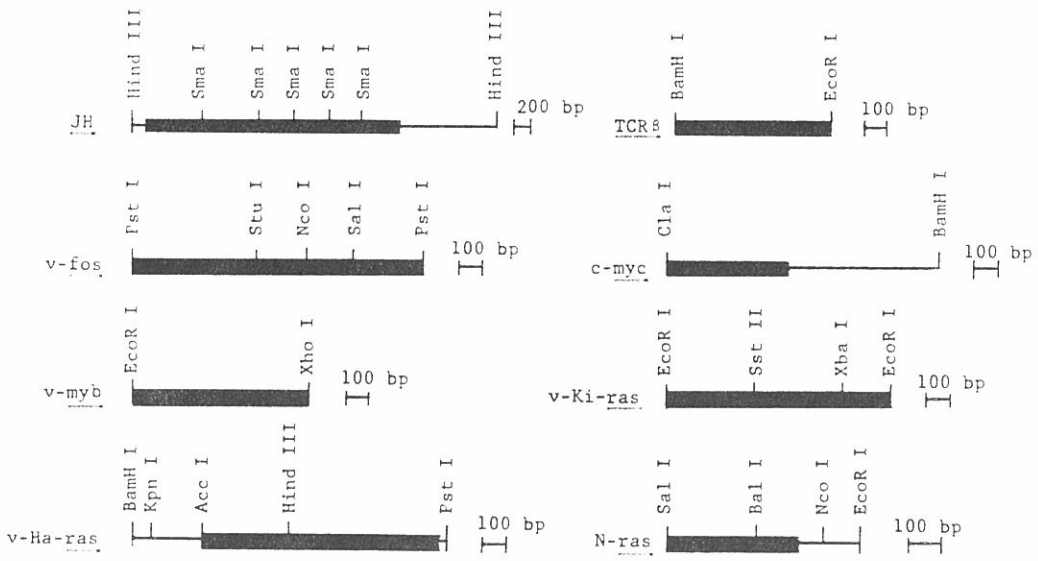

Figure 1. DNA probes used for in situ hybridization.

Labeling of DNA with photobiotin acetate

In very subdued light, 1 mg/ml of photobiotin acetate in distilled water was added to an equal volume of DNA (0.5–1.0 mg/ml) in a Eppendorf tube. The tube was placed in crushed ice with the lid open and irradiated for 20 minutes 10 cm beneath a 500 W mercury reflector lamp. A buffer of 0.1 M Tris-HCl (pH 9.0), 1.0 mM EDTA was added to the reaction mixture to a final volume of 100 μl, then was extracted twice with 2-butanol. After the addition of carrier tRNA, biotin-labeled DNA was sedimented by ethanol precipitation, washed once with cold 70% ethanol, dried in a vacuum, and dissolved in 50 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE, pH 8.0).

In situ hybridization

Pretreatment[2,4]: Ten micrometers of frozen tissue sections on 0.25% gelatin, 0.25% chrome alum-treated slides was fixed for 15 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.1% glutaraldehyde at room temperature. After fixation, sections were washed twice with PBS, immersed in 0.2 N HCl for 15 minutes at room temperature, and then washed twice with PBS. Sections were then incubated in 2 × SSC (1 × SSC; 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide for 20 minutes at 60°C, washed again with PBS, and treated with 2.5 μg/ml of proteinase K in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (TE, pH 7.5) for 15 minutes at 37°C. Following washing with PBS twice, sections were postfixed in 4% paraformaldehyde for three minutes at room temperature, washed twice with PBS, and treated with 0.25% acetic anhydride. After a PBS wash, they were dehydrated in a graded series (70%, 95%, 100%) of ethanol, then air-dried.

Prehybridization[2]: A mixture of sonicated salmon sperm DNA, yeast tRNA, and polyadenylate was boiled for five minutes and chilled on ice before preparation of the final prehybridization solution. The final prehybridization solution consisted of 50% deionized formamide, 2 × SSC, 4 × Denhardt's solution (1 × Denhardt's solution; 0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone), 20 mM Vanadyl ribonucleoside complex, 1 mg/ml of sonicated salmon DNA, 1 mg/ml of tRNA, and 200 μg/ml of polyadenylate. Tissue sections on the slides were covered with 100 μl of prehybridization solution and incubated at 42°C for three hours in a humidity chamber.

Hybridization[4]: The probe DNA, sonicated salmon sperm DNA, yeast tRNA, and polyadenylate were boiled for five minutes, chilled on ice, then transferred to the hybridization mixture; thus, the final hybridization solution contained 45% deionized formamide, 2 × SSC, 10% sodium dextran sulfate, 1 × Denhardt's solution, 2 mM Vanadyl ribonucleoside complex, 500 μg/ml of sonicated salmon sperm DNA, 500 μg/ml of tRNA, 100 μg/ml of polyadenylate, and 1 μg/ml of probe DNA. After the prehybridization solution was removed by suction, the hybridization solution was applied to the sections, covered with a glass coverslip, and the edges sealed with rubber cement. The slides were incubated in a humidity chamber for 12–16 hours at 42°C.

Washing: After hybridization, coverslips were gently removed from slides by soaking each slide in 2 × SSC. Slides were washed once while shaking in 50% formamide, 2 × SSC, for 30 minutes at 37°C and then twice in 50% formamide, 1 × SSC, for 30 minutes each time at 37°C. They were again washed twice while shaking in 0.1 × SSC for 30 minutes at 37°C–40°C.

Colorimetric detection: Slides were processed using BluGENE (BRL, Gaithersburg, Md) with certain modifications to the recommended protocol. The slides were immersed in 1% skim milk in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl (buffer-1) for one hour at 37°C to block nonspecific streptavidin-alkaline

phosphatase binding. After removal of excess buffer with Kimwipes, sections on the slide were covered with 1 μg/ml of streptavidin-alkaline phosphatase in buffer-1 and incubated for one hour at 37°C in a humidity chamber. Slides were then washed twice in buffer-1 for 10 minutes each time while gently shaking, then twice in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM M_gCl_2 (buffer-2) for five minutes each time. The substrate solution contained 33 μl of nitroblue tetrazolium (75 mg/ml in 70% dimethylformamide) and 25 μl of 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml in dimethylformamide) in 7.5 ml of buffer-2. For color development, slides were incubated in the dark at room temperature with substrate solution for one to two hours. After development, the slides were immersed in PBS, counterstained with 0.5% methylgreen, and mounted with Perma Fluor (Nippon Tanner, Tokyo).

Total RNA extraction[14,15]

Lymphoma tissues, about 0.8 g in wet weight, were finely cut with a pair of scissors, then sufficiently homogenized in 8 ml of 4 M guanidine thiocyanate, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol, 5 mM sodium citrate (pH 7.0) in a Teflon-glass homogenizer. After shearing of the DNA by several passages through a 21G needle, the homogenate was layered onto a 0.5 volume of 5.7 M CsCl, 0.1 M EDTA (pH 7.5), then centrifuged at 140,000 × g for 15 hours at 15°C. The resulting pellets were resuspended in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS) and extracted twice with phenol. The aqueous phase was extracted once with chloroform-phenol (1:1, v/v), then once with chloroform. The aqueous phase was mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.2 volume of ethanol, then stored at −20°C for at least two hours. Following RNA sedimentation by centrifugation for 15 minutes at 12,000 rpm, RNA was washed with 90% ethanol and stored in 70% ethanol at −70°C.

Dot-blot hybridization

Nitrocellulose filter (BA85; Schleicher & Schuell, Inc., Dassel, FRG) was soaked first in distilled water, then thoroughly in 5 × SSC, and air-dried. The RNA was resuspended in 7.4% formaldehyde, 5 × SSC, and incubated at 60°C for 15 minutes.[16] Using suction, 100 μl of each sample was applied to a nitrocellulose filter with dot-blotting apparatus for dot uniformity. RNA-treated filters were dried in a vacuum oven at 80°C for two hours, then prehybridized in 50% deionized formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM sodium phosphate (pH 6.5), and 500 μg/ml of sonicated denatured salmon sperm DNA for 3–12 hours at 43°C. Hybridization was carried out while shaking in a water bath at 43°C for 18–24 hours in a buffer containing 45% deionized formamide, 5 × SSC, 10% sodium dextran sulfate, 1 × Denhardt's solution, 20 mM sodium phosphate (pH 6.5), 200 μg/ml of sonicated denatured salmon sperm DNA, 20 μg/ml of polyadenylate, and 250–500 ng/ml of biotin-labeled DNA probe. Filters were then washed once in 2 × SSC/0.1% SDS for 10 minutes at room temperature, twice in 1 × SSC/0.1% SDS for 10 minutes each time, and twice in 0.1 × SSC/0.1% SDS for one hour each time at 58°C–60°C. Colorimetric detection was carried out in the same way described in the in situ hybridization section except that filters were

incubated in 3% skim milk at 65°C for one hour to block nonspecific binding of streptavidin-alkaline phosphatase.

Results

Expression of *TCRβ* and *IgH* genes in NHL

Expression of *TCRβ* and *IgH* genes in NHL was analyzed using in situ hybridization assays. The results obtained under blind conditions were compared with NHL typing by T and B cell surface markers. Examples of in situ hybridization are shown in Figure 2 and analyses of 43 cases are summarized in Table 1. Out of 21 cases of B cell-type NHL, 16 cases were strongly positive for *JH* expression whereas none tested strongly positive for expression of *TCRβ* gene. On the contrary, 15 out of 22 cases of T cell-type NHL were strongly positive for *TCRβ* expression, and 7 cases were weakly positive. Regarding the expression of *JH,* 5 cases were faintly positive and the remaining 17 cases were negative. These results, as a whole, show that the expression of *TCRβ* and *IgH* genes detected by in situ hybridization is compatible with typing by cell surface markers.

Expression of proto-oncogenes in NHL

The expression of *fos, myc, myb,* Ki-*ras,* Ha-*ras,* and N-*ras* in NHL was analyzed using in situ hybridization assays. Examples of the analysis are shown in Figures 3 and 4, and the results are summarized in Table 2. It was observed that in B cell-type NHL, 80%–90% of the total cases expressed *fos, myc,* and *myb* whereas only 9%–33% expressed Ki-*ras,* Ha-*ras,* and N-*ras.* In T cell-type NHL, however, 59%–77% of the total cases expressed *fos, myc, myb,* and Ki-*ras.* As shown in Figures 3 and 4, proto-oncogene action was, when expressed, only diffusely recognizable in each sample with no particular pattern of localization. In most samples demonstrating positive reactivity, virtually all cells were positive when observed under high magnification. Such was the case for all proto-oncogenes examined in this study.

Comparative analysis of proto-oncogene expression by in situ hybridization and dot hybridization

In order to further examine the specificity of in situ hybridization, total cellular RNA was extracted from selected cases of NHL and also analyzed using dot hybridization assays. Examples of dot hybridization assays shown in Figure 5 are compared with the results of in situ hybridization. Good correlation can be seen between the results of the two assay systems.

Expression of proto-oncogenes in histopathologically classified NHL

Cases of NHL were histopathologically classified based on the International Working Formulation, and the classification types were compared with expression of the various proto-oncogenes. The results are shown in Table 3. Though no clear association was observed in general, expression of Ki-*ras* was slightly more frequent in large cell (immunoblastic)-type NHL than in other types.

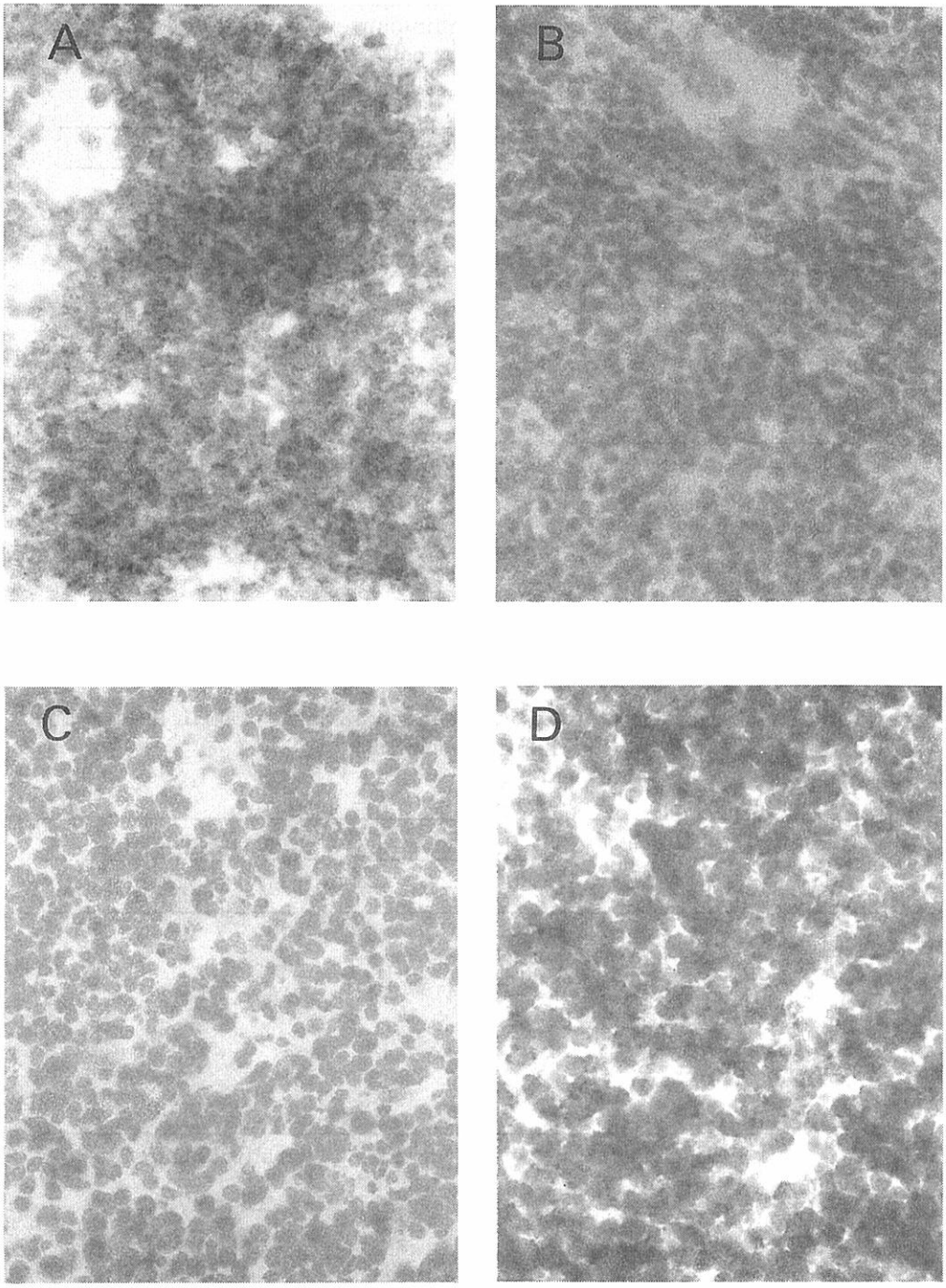

Figure 2. Examples of in situ hybridization with JH *and* TCRβ. *B cell-type lymphoma (2A, 2B) and T cell-type lymphoma (2C, 2D) were analyzed with* JH *(2A, 2C) or* TCRβ *(2B, 2D).*
Original magnification: $\times$ *200.*

Table 1. Expression of *TCRβ* and *IgH* genes in NHL as detected by in situ hybridization: A summary of 43 cases and a comparison with typing by cell surface markers

Type of NHL by surface markers	Grade of expression*	Expression of *TCRβ and JH*	
		TCRβ	*JH*
B cell-type	+ ~ ++	0**	16
(21 cases)***	±	2	5
	−	19	0
T cell-type	+ ~ ++	15	0
(22 cases)	±	7	5
	−	0	17

* + ~ ++, strongly positive; ±, weakly positive; −, negative (Figure 3)
** Number of cases
*** Total number of cases

Table 2. Expression of proto-oncogenes in 43 cases of NHL analyzed by in situ hybridization

Type of NHL by surface markers	Grade of expression*	Expression of proto-oncogene					
		fos	*myc*	*myb*	Ki-*ras*	Ha-*ras*	N-*ras*
B cell-type	+ ~ ++	13**	11	10	5	2	2
(21 cases)***	±	6	6	7	2	3	0
	−	2	4	4	14	16	19
T cell-type	+ ~ ++	11	8	10	8	2	0
(22 cases)	±	6	6	3	7	5	3
	−	5	8	9	7	15	19

* + ~ ++, strongly positive; ±, weakly positive; −, negative (Figure 3)
** Number of cases
*** Total number of cases

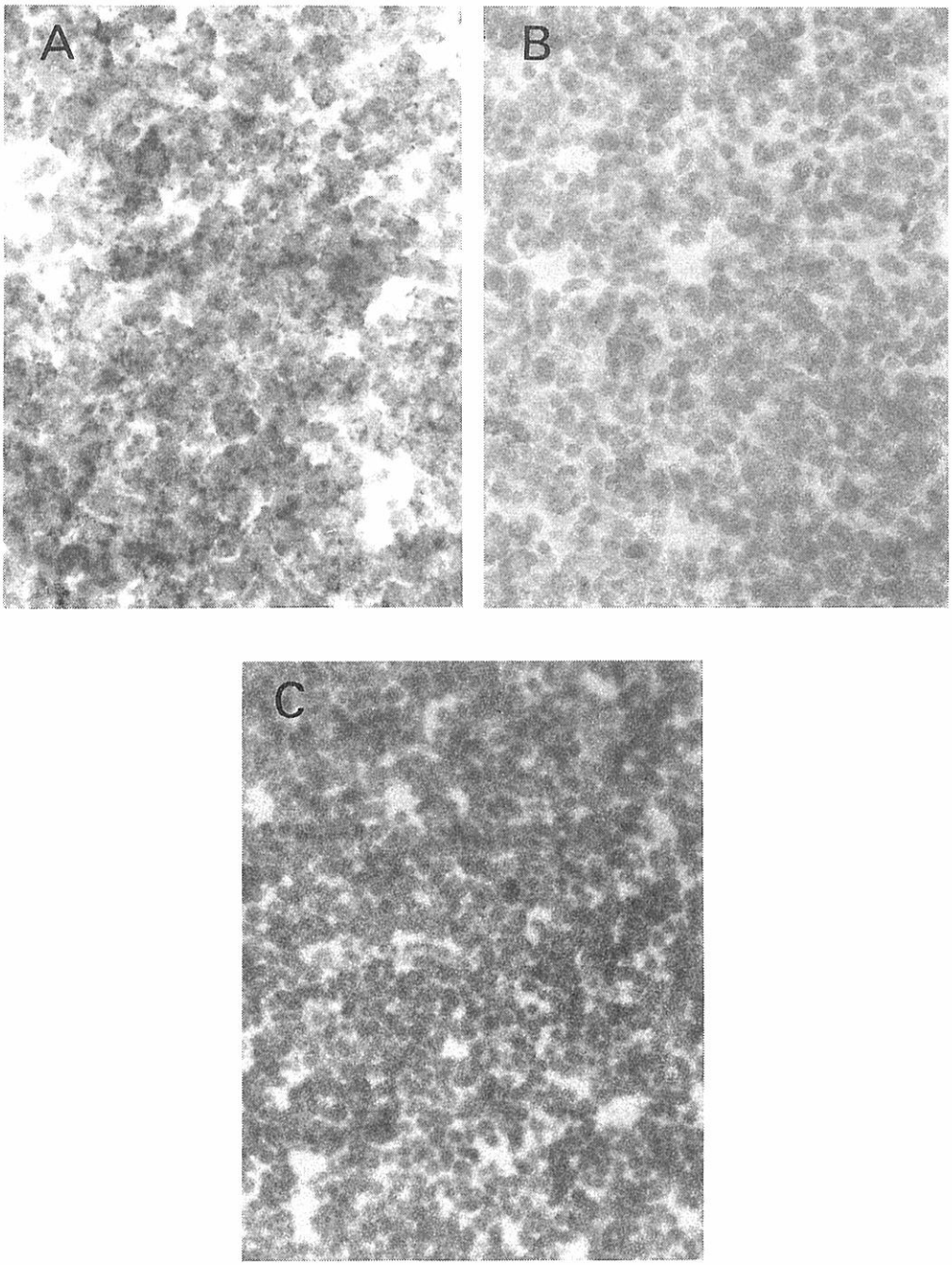

*Figure 3. Intensity grading of reactivities in the in situ hybridization. Three cases of NHL were analyzed with v-*fos *probes and scored as follows: 3A, (+∼++); 3B, (±); 3C, (−). Original magnification: × 200.*

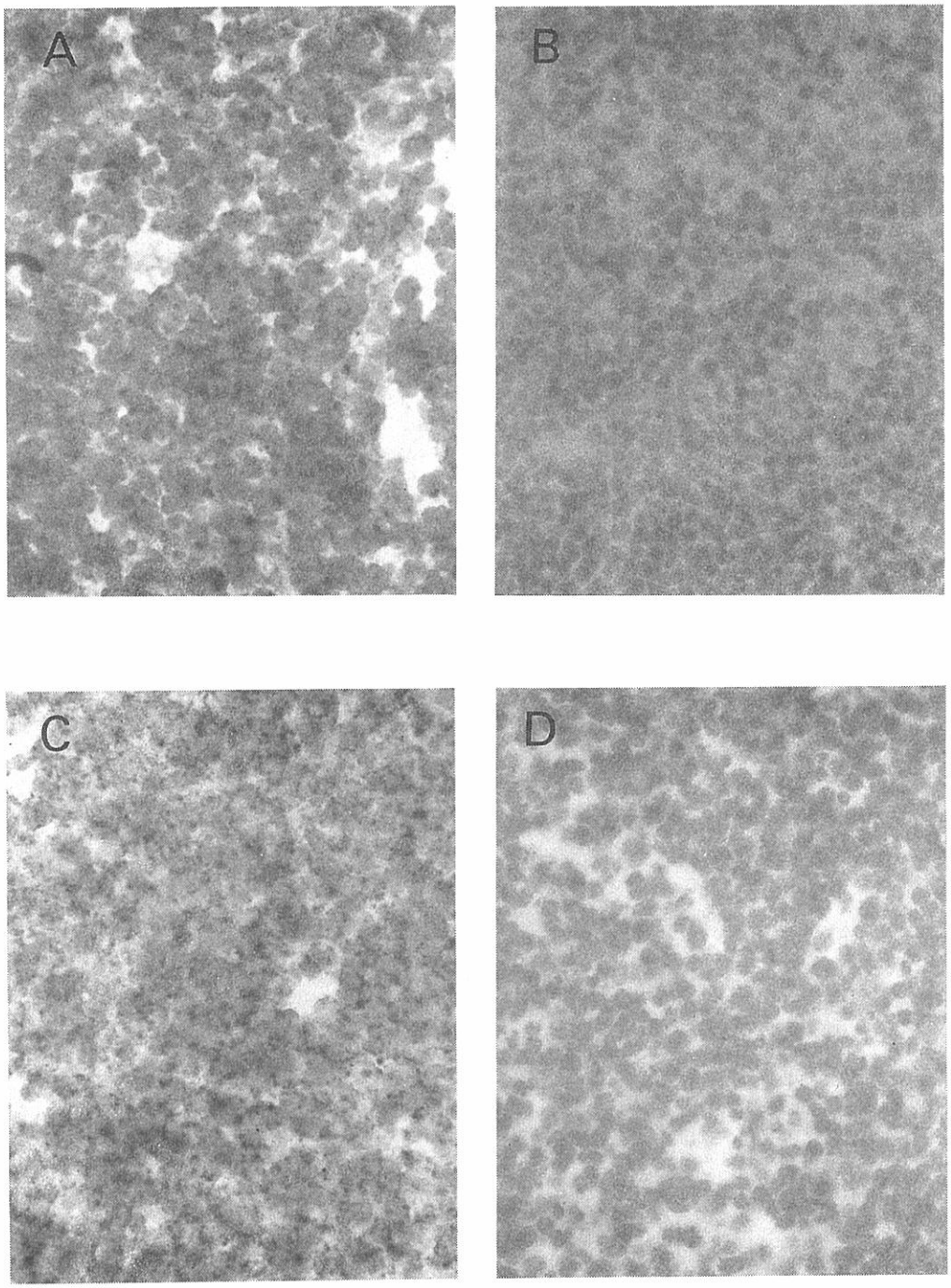

Figure 4. Examples of in situ hybridization with c-myc *(4A, 4B),* v-myb *(4C, 4D), and* v-Ki-ras *(4E, 4F). 4A, 4C, and 4E were scored as (+∼++), whereas 4B, 4D, and 4F were scored as (−).*
Original magnification: × *200.*

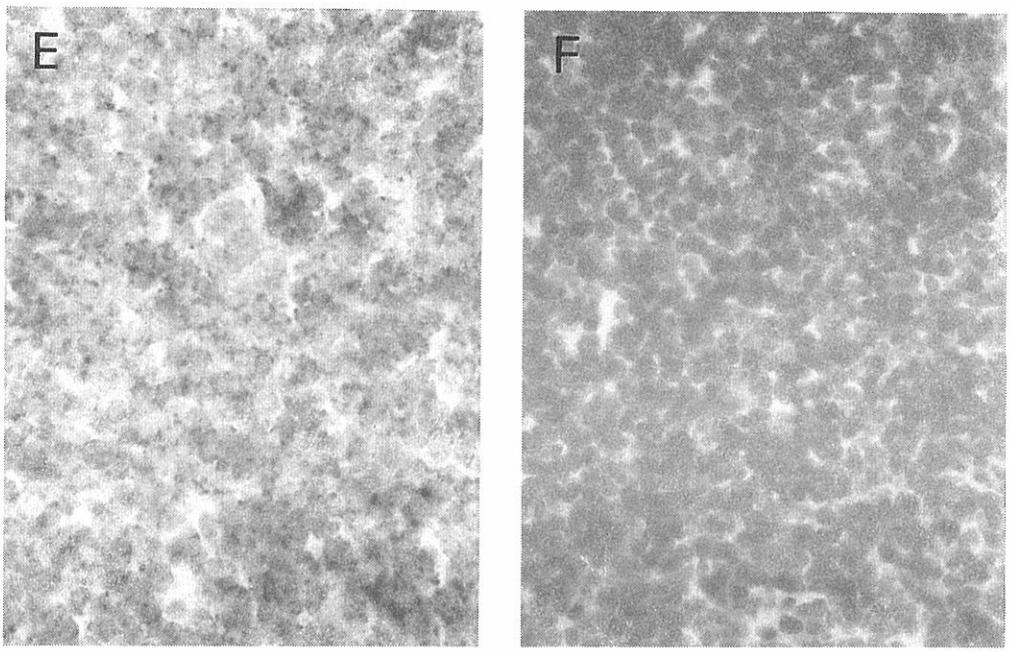

Figure 4. Continued

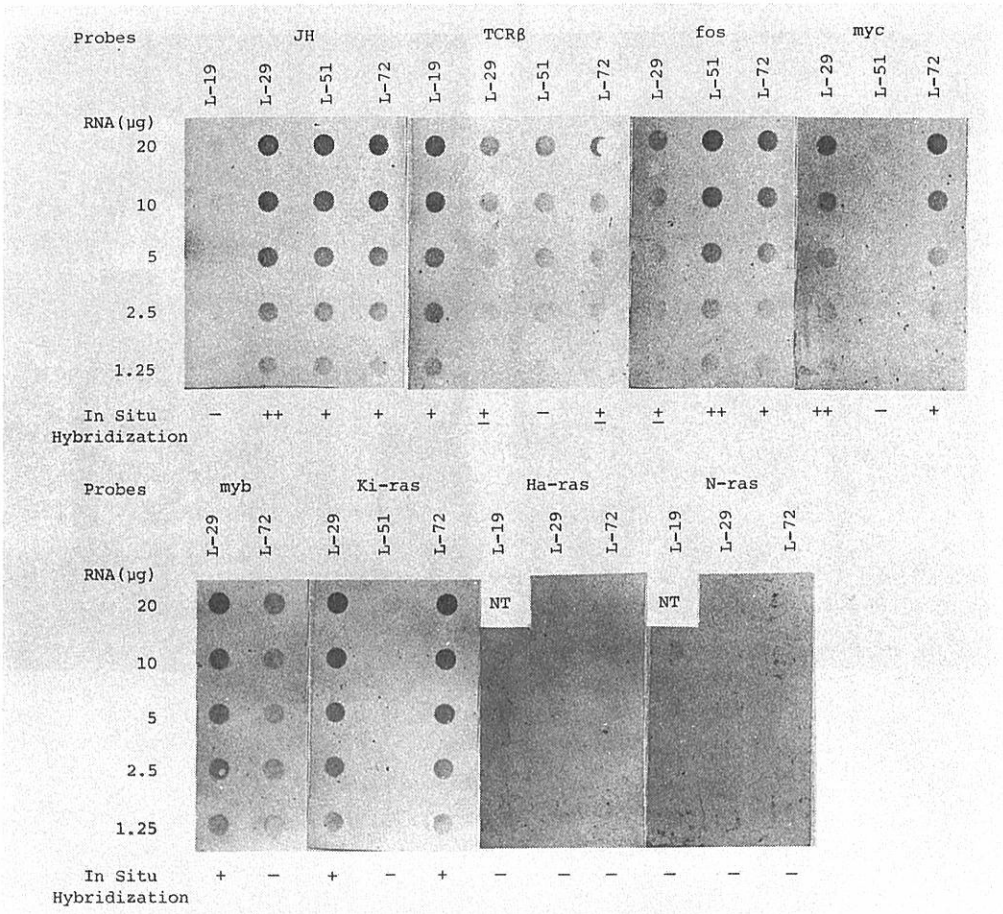

Figure 5. Comparison of results of dot hybridization assay with those of in situ hybridization. RNA was extracted from four cases of NHL (L-19, L-29, L-51, and L-72) as described in the Materials and Methods section, and dot hybridization was performed with the listed DNA probes. Results of in situ hybridization assay correlated well with those of dot hybridization assay. For some DNA probes, fewer cases were analyzed by dot hybridization because of the limited amount of RNA available; NT, not tested.

Table 3. Expression of proto-oncogenes in histopathologically classified NHL

Histological type*	No. of cases studied	No. of cases with positive expression**					
		fos	*myc*	*myb*	Ki-*ras*	Ha-*ras*	N-*ras*
Low-grade malignancy							
Follicular***	3	3	3	2	1	1	0
Small Lymphocytic	2	2	2	1	1	1	0
Intermediate malignancy							
Diffuse small cleaved	4	4	4	4	2	2	1
Diffuse mixed	7	6	4	5	3	2	1
Diffuse large cell	8	7	5	6	3	2	2
High-grade malignancy							
Large cell immunoblastic****	16	11	10	9	10	3	1
Lymphoblastic	3	3	3	3	2	1	0

* According to the International Working Formulation[9]

** Cases scored as ± ~ ++ were classified as positive

*** Mixed type in all three cases

**** Six cases with polymorphous morphology

Discussion

In an attempt to use in situ hybridization for proto-oncogene expression, it was most essential to establish the specificity of this methodology. We believe the findings of this study sufficiently justify the specificity of this in situ hybridization technique. Expression of both *TCRβ* and *IgH* genes was extremely compatible with the results of immunological typings using cell surface markers. Whole typing by in situ hybridization was performed as previously described under totally blind conditions. In all probes used in this study, positive reactions were observed in cytoplasma of NHL, as shown in Figures 2–4. No reactivity was observed when photobiotinylated pBR322 alone was used as the negative control. The results of in situ hybridization were also remarkably compatible with those of dot hybridization performed with RNA extracted from several samples. Furthermore, all results demonstrated a high degree of reproducibility.

It is necessary to comment on several technical points. Firstly, the pretreatment of samples on glass slides with an optimal concentration of proteinase-K seemed to be critical. Treatment with proteinase-K of either too high or too low a concentration resulted in fainter signals. Secondly, prehybridization significantly reduced nonspecific binding of the probes, though not drastically. Thirdly, in addition to the washing conditions described by Lawrence and Singer,[4] it was necessary to wash samples in 0.1 × SSC, accompanied by vigorous shaking at 37°C–40°C for at least one hour.

Among the six proto-oncogenes studied, expression of *fos, myc,* and *myb* was observed in 59%–77% of T cell-type NHL and 80%–90% of B cell-type NHL regardless of histology or malignancy grade. More than two of these proto-oncogenes were co-expressed in 79% of the total cases, and only one case of NHL expressed none of these three proto-oncogenes. In contrast, expression of the *ras* gene family, in particular Ha-*ras* and N-*ras,* was observed in a rather limited number of cases, but in T cell-type NHL or large cell-type (immunoblastic) NHL Ki-*ras* gene was more frequently expressed. Malignant cells of each case showed uniformly positive or negative expression of genes detected by these probes. Since expression of these proto-oncogenes is either totally negative or extremely weak in normal lymph nodes (data not shown), the presence of proto-oncogene-expressing cell localization in normal lymph nodes remains to be elucidated.

Roy-Burman et al[17] studied the expression of c-*myc,* c-*myb* and c-*erb* B in 17 cases of NHL using dot hybridization assays. According to them, expression of c-*myc* was noticed in essentially all cases while that of c-*myb* was noticed in only six. Slamon et al[18] analyzed 15 different oncogenes in a series of tumors including two cases of NHL. The results showed moderate expression of c-*fos* and c-*myc,* and high expression of c-Ha-*ras.* Expression of c-*myb* and c-Ki-*ras* proved variable. When Westin et al[19] analyzed two Burkitt's lymphoma lines and EBV-transformed cell lines, c-*myc* and c-Ha-*ras* were moderately expressed though there was no expression of c-*myb.* These results as well as our study indicate that expression of nuclear proto-oncogenes is in general frequent in NHL.

All *fos, myc,* and *myb* proto-oncogenes, often expressed in NHL, encode nuclear proteins. Various experimental evidence indicates that all of these nuclear proteins are involved in the regulation of cellular metabolism in connection with DNA replication or transcriptional mechanisms.[20–24] Induction of cellular proliferation through various methods often leads to an increase in the cellular transcripts of these proto-oncogenes and possibly their gene products,[25–29] while the induction of cellular differentiation often results in their decrease.[30–32] We are, therefore, inclined to consider that a high frequency of elevated *fos, myc,* and *myb* expression is a reflection of the highly proliferative characteristics of NHL.

In this study, expression of *ras* genes was rather infrequent in NHL. Expression of the Ki-*ras* gene, however, seemed to be more frequent in T cell-type NHL than in B cell-type NHL, as shown in Table 2. This was particularly the case in adult T cell leukemia/lymphoma, hence Ki-*ras* gene expression was relatively frequent in large cell-type (immunoblastic) NHL, most of which are adult T cell leukemia/lymphoma in the current analysis. It is important to determine whether activation of *ras* genes is associated with elevated gene expression in NHL, hence analysis is ongoing in our laboratory.

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