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Detection of the c-*myc* Gene Product in Urinary Bladder Cancer

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Expression of the c-*myc* gene product in urinary bladder cancer was investigated by immunohistochemical staining with anti-c-*myc* monoclonal antibody (mAb) MYC-1. Positive staining was observed in the cytoplasm, but not in the nucleus in tissues fixed with 10% formalin. On the other hand, positive staining was localized in the nucleus in cryopreserved tissues. Of 34 cryopreserved specimens examined, positive staining with MYC-1 mAb was observed in 1 of 12 (8.3%) of grade 1 (G1), 12 of 15 (80%) of G2 and 6 of 7 (86%) of G3. Positive staining with Ki-67 mAb was observed in 2 of 12 (17%) of G1, 12 of 15 (80%) of G2, and 6 of 7 (86%) of G3. These results suggest that tumors with higher nuclear pleomorphism contain more proliferating cells.

Key words: c-*myc* — Immunohistochemistry — Bladder cancer — Nuclear pleomorphism

Expression of the c-*myc* gene product has been shown to be associated with cell proliferation. Transcription of the c-*myc* gene was initiated in T-lymphocytes immediately after the addition of mitogen[1] and DNA synthesis or blast transformation was inhibited by the addition of anti-c-*myc* protein antibody[2] or complementary oligonucleotide of the c-*myc* gene.[3]

We recently demonstrated that the frequency of a positive reaction with anti-c-*myc* monoclonal antibody (mAb) MYC-1 was correlated with the grade of nuclear pleomorphism in primary and metastatic renal cell carcinomas.[4] This finding suggested that renal cell carcinomas with a higher grade of nuclear pleomorphism contain more proliferating cells. In this study, for further investigation of the relationship between the expression of the c-*myc* gene and nuclear pleomorphism, we extended our analyses to urinary bladder tumors.

Specimens of primary bladder cancer (transitional cell carcinoma) and normal bladder tissue were obtained at surgery. The tissues were quickly embedded in OCT compound and stored at $-80\,^{\circ}C$. MYC-1 mAb was prepared from mice immunized with a truncated c-*myc* protein, Mr 23,000 protein, produced in *Escherichia coli* transfected with the pTR *myc* 10 gene. The specificity of MYC-1 mAb has been reported.[5] Briefly, in enzyme-linked immunosorbent assay, MYC-1 mAb reacted with Mr 23,000 protein, a product of the truncated c-*myc* gene, but not with Mr 21,000 protein, a product of the *ras* gene, the 5′ end of which is a constituent of the pTR *myc* 10 gene. Moreover, addition of Mr 23,000 protein specifically inhibited the reaction of MYC-1 mAb with nuclear extracts of HL-60 cells. In Western blotting, MYC-1 mAb reacted only with Mr 58,000 and 60,000 molecules of nuclear extracts of the HL-60 and Colo 320 cell lines and application of MYC-1 mAb to a Sepharose 4B column coupled with the Mr 23,000 protein reduced the reaction.[5] Ki-67 mAb, prepared from mice immunized with the crude nuclear fraction of L428 cells derived from a patient with Hodgkin's disease,[6] was purchased from Dako (Uppsala). It recognized a nuclear antigen present in proliferating cells, but not in resting cells. RASK-3 mAb, prepared as described elsewhere,[7] reacted with the *ras* gene product. The indirect immunoperoxidase method with a Vectastain avidin-biotin complex kit for mouse IgG (Vector Laboratories, Burlingame, CA) was used with the following modifications. Cryopreserved tissue was sectioned on a cryostat and fixed in 4% Lillie's buffered formalin for 15 min at room temperature. Slides were blocked with 0.5% normal horse serum for 30 min and then incubated with mAbs for 60 min at room temperature. They were then washed extensively with phosphate-buffered saline, and endogenous peroxidase activity was blocked by treatment with 0.6% H_2O_2 in methanol for 30 min. Ascites from mice bearing NS-1 myeloma cells or normal mouse serum was routinely included as a negative control. Specimens in which more than 5% of the cells in microscopic fields were stained were regarded as positive. Histopathological grading was determined according to the general code for clinical and pathological studies on bladder cancer of the Japanese Urological Association.[8]

For immunohistochemical detection of the c-*myc* gene product, we first studied suitable conditions of preservation and fixation of tissues. As shown in Fig. 1, in paraffin-embedded sections, which are routinely used for histopathological examination, positive staining was ob-

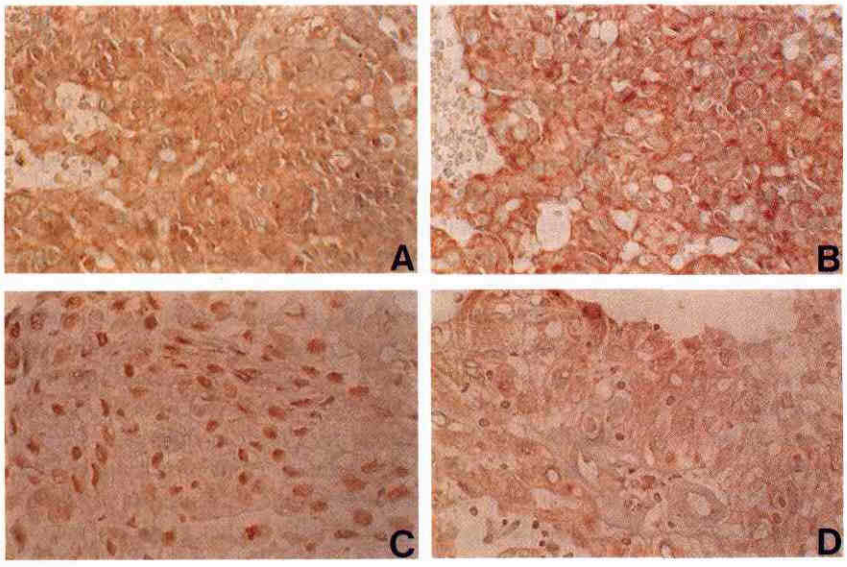

Fig. 1. Immunohistochemical staining with MYC-1 mAb (A, C) and RASK-3 mAb (B, D) of bladder cancer in sections embedded in paraffin (A, B) and cryopreserved sections (C, D). The c-*myc* protein is not detected in the nucleus in the section embedded in paraffin (A), but positive staining is observed in the nucleus in the cryopreserved section (C).

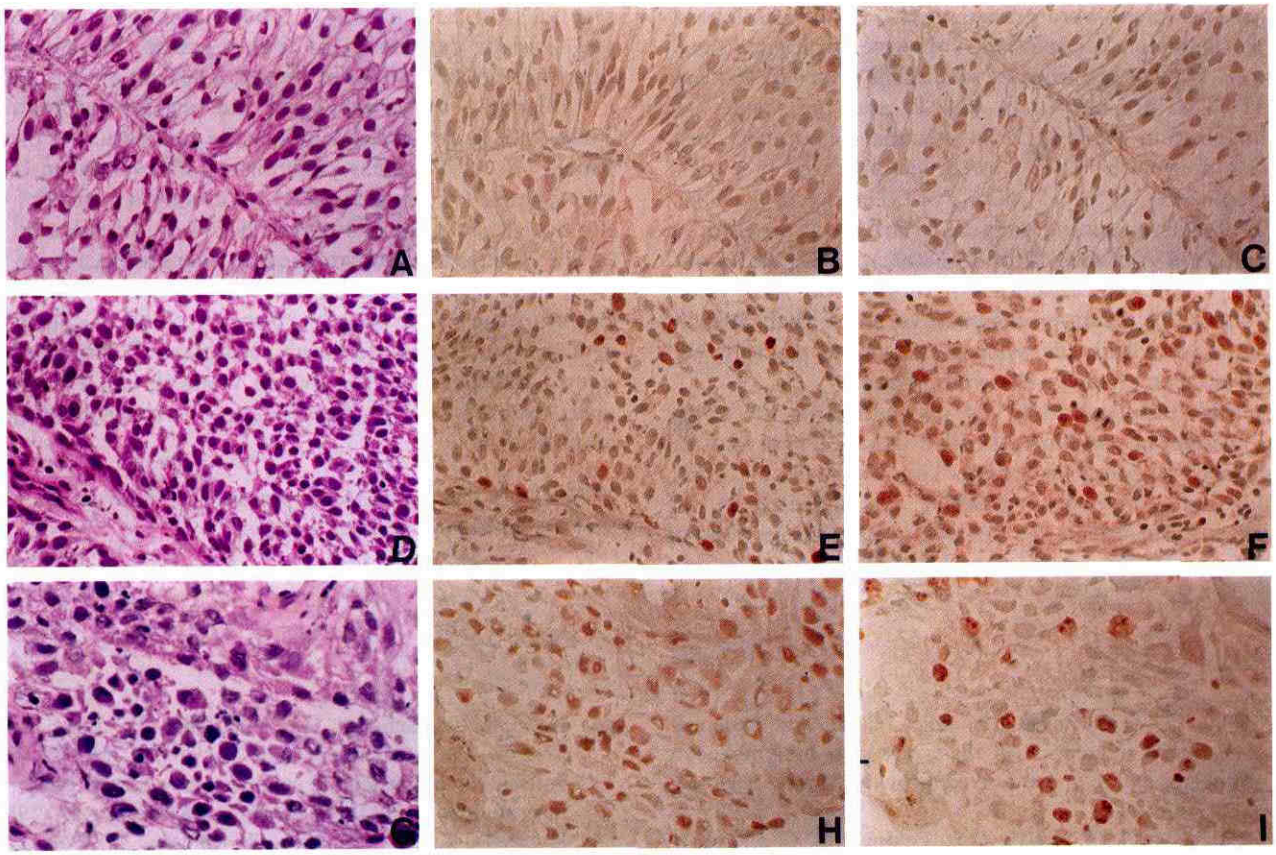

Fig. 2. Immunohistochemical stainings with MYC-1 mAb (B, E, H) and Ki-67 mAb (C, F, I) and hematoxylin-eosin stainings (A, D, G) of bladder cancer of grade 1 (A–C), grade 2 (D–F) and grade 3 (G–I).

Table I. Correlation of c-*myc* Expression and Ki-67 mAb Reactivity with Histopathological Grading in Bladder Cancer

mAb	Positive staining (%)			
	G1	G2	G3	Total
MYC-1	1/12 (8)	12/15 (80)	6/7 (86)	19/34 (56)
Ki-67	2/12 (17)	12/15 (80)	6/7 (86)	20/34 (59)

served in the cytoplasm, but not in the nucleus of bladder cancer cells. On the other hand, in cryopreserved sections, positive staining was localized in the nucleus. Because the c-*myc* gene product has been shown to be a nuclear DNA binding protein,[9, 10] cryopreserved tissue was used for detection of the c-*myc* gene product in subsequent analyses. The staining patterns of these two types of preparations with RASK-3 mAb, which has been shown to detect N-, Ha-, K-*ras* gene products,[9] were not significantly different.

We then investigated whether there was any correlation between the reactivity with MYC-1 mAb and the histopathological grade of bladder cancer. Bladder cancer specimens were graded as G1, G2 or G3 according to the general code of the Japanese Urological Association. Typical stainings of specimens with MYC-1 and Ki-67 mAb are shown in Fig. 2, and the results are summarized in Table I. Of 34 specimens examined, positive staining with MYC-1 mAb was observed in 1 of 12 (8.3%) of G1, 12 of 15 (80%) of G2 and 6 of 7 (86%) of G3. Positive staining with Ki-67 mAb was observed in 2 of 12 (17%) of G1, 12 of 15 (80%) of G2, and 6 of 7 (86%) of G3. The reactions of these mAbs were clear-cut: when the reaction was positive, more than 30% of the cells were stained, whereas when the reaction was negative, scarcely any cells were stained.

In this study, we observed dislocation of the c-*myc* gene product in paraffin-embedded tissues. These findings confirmed our previous findings[4] on renal cell carcinoma tissue. The c-*myc* gene product was detected in the cytoplasm, but not in the nucleus in paraffin sections of 10% formalin-fixed specimens by immunohistochemical staining with mouse mAb[11, 12] or rabbit antiserum[13] prepared against a synthetic peptide. The absence of the c-*myc* gene product in the nucleus thus appears to be due to inappropriate conditions of preservation and/or fixation of the tissue.

The nuclear grade has been used as an indicator of malignancy and is correlated with the prognosis of patients with carcinomas of the urinary bladder. In this study, we demonstrated that expression of the c-*myc* gene product was correlated with the nuclear grade of bladder cancer. Reactivity with Ki-67 mAb, which also recognizes a nuclear protein associated with cell proliferation, was also correlated with the grade of nuclear pleomorphism. These results are entirely consistent with our previous finding that expression of the c-*myc* gene product is correlated with nuclear pleomorphism of renal cell carcinomas. The results suggest that carcinomas of the urinary bladder and renal cells with higher grades of nuclear pleomorphism contain more proliferative cells.

Renal cell carcinoma originates in proximal tubular epithelium and bladder carcinoma originates in transitional cell epithelium. There is also a difference in the clinical courses of these two tumors. However, Fradet *et al.*[14] recently demonstrated that T43 (URO-10) mAb reacted with both proximal tubule, renal cell carcinoma and bladder carcinoma of higher grade, but not with other normal or malignant tissues. These findings suggest a similarity between renal cell and bladder carcinomas. Therefore, to determine whether the correlation of c-*myc* expression with nuclear pleomorphism observed in these two types of tumors is caused by a common nature of the tumors, other types of tumors should be investigated.

The genetic mechanism causing over-expression of the c-*myc* gene in the tissues examined is unknown. Amplification of the c-*myc* gene has been observed in colorectal cancers[15] and small cell lung carcinoma.[16] However, in one of these studies,[16] elevated transcript of the c-*myc* gene was frequently observed in the absence of gene amplification.

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REFERENCES

1) Kelly, K., Cochran, B. H., Stiles, C. D. and Leder, P. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factors. *Cell*, **35**, 603–610 (1983).
2) Studzinski, G. P., Brelvi, Z. S., Feldman, S. C. and Watt, R. A. Participation of c-*myc* protein in DNA synthesis of human cells. *Science*, **234**, 467–470 (1986).
3) Heikkila, R., Schwab, G., Wickstrom, E., Loke, S. L., Pluznik, D. H., Watt, R. and Neckers, L. M. A c-*myc* antisense oligonucleotide inhibits entry into S phase but not progress from G_0 to G_1. *Nature*, **328**, 445–449 (1987).
4) Kinouchi, T., Saiki, S., Naoe, T., Uenaka, A., Kotake, T., Shiku, H. and Nakayama, E. Correlation of c-*myc* expression with nuclear pleomorphism in human renal cell carci-

noma. *Cancer Res.*, **49**, 3627–3630 (1989).
5) Naoe, T., Nozaki, N., Yamada, K., Okazaki, T., Nakayama, E., Kurosawa, Y. and Shiku, H. Diversity of cellular molecules in human cells detected by monoclonal antibodies reactive with c-*myc* proteins produced in *Escherichia coli*. *Jpn. J. Cancer Res.*, **80**, 747–753 (1989).
6) Gerdes, J., Schwab, U., Lemke, H. and Stein, H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int. J. Cancer*, **31**, 13–20 (1983).
7) Yoshida, K., Hamatani, K., Koide, H., Ikeda, H., Nakamura, N., Akiyama, M., Tsuchiyama, H., Nakayama, E. and Shiku, H. Preparation of anti-*ras* Mr 21,000 protein monoclonal antibodies and immunohistochemical analyses on expression of ras genes in human stomach and thyroid cancers. *Cancer Res.*, **48**, 5503–5509 (1988).
8) Japanese Urological Association and Japanese Pathological Society. "General Rules for Clinical and Pathological Studies on Bladder Cancer," Ed. 1 (1980). Kanehara Co., Ltd., Tokyo (in Japanese).
9) Donner, P., Wilke, I. G. and Moelling, K. Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. *Nature*, **296**, 262–266 (1982).
10) Alitalo, K., Ramsay, G., Bishop, J. M., Pfeifer, S. O., Colby, W. W. and Levinson, A. D. Identification of nuclear proteins encoded by viral and cellular *myc* oncogenes. *Nature*, **306**, 274–277 (1983).
11) Stewart, J., Evan, G., Watson, J. and Sikora, K. Detection of the c-*myc* oncogene product in colonic polyps and carcinomas. *Br. J. Cancer*, **53**, 1–6 (1986).
12) Sikora, K., Evan, G., Stewart, J. and Watson, J. V. Detection of the c-*myc* oncogene product in testicular cancer. *Br. J. Cancer*, **52**, 171–176 (1985).
13) Iwanaga, T., Fujita, T., Tsuchihashi, T., Yamaguchi, K., Abe, K. and Yanaihara, N. Immunocytochemical detection of the c-*myc* oncogene product in human fetuses. *Biomed. Res.*, **7**, 161–166 (1986).
14) Fradet, Y., Cordon-Cardo, C., Thomson, T., Daly, M. E., Whitmore, W. F., Jr., Lloyd, K. O., Melamed, M. R. and Old, L. J. Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, **81**, 224–228 (1984).
15) Sikora, K., Chan, S., Evan, G., Gabra, H., Markham, N., Stewart, J. and Watson, J. c-*myc* oncogene expression in colorectal cancer. *Cancer*, **59**, 1289–1295 (1987).
16) Takahashi, T., Obata, Y., Sekido, Y., Hida, T., Ueda, R., Watanabe, H., Ariyoshi, Y., Sugiura, T. and Takahashi, T. Expression and amplification of *myc* gene family in small cell lung cancer and its relation to biological characteristics. *Cancer Res.*, **49**, 2683–2688 (1989).