Nagoya J. Med. Sci. **55.** 125 ~ 130, 1993

EXPRESSION OF PROTO-ONCOGENES AND TUMOR SUPPRESSOR GENES IN *IN VITRO* CELL LINES DERIVED FROM A THYMUS, THYMOMA, AND MALIGNANT THYMOMA OF RATS

YOSHIHISA SAKAI[1], WORAWIDH WAJJWALKU[1], MASAHIDE TAKAHASHI[1], AKIRA MASUDA[2], KAZUHIKO R. UTSUMI[2], and MUTSUSHI MATSUYAMA[1]

[1]*Department of Pathology, Nagoya University School of Medicine, Nagoya, Japan and* [2]*Laboratory of Ultrastructure Research, Aichi Cancer Center Research Institute, Nagoya, Japan*

ABSTRACT

To analyze the processes of the development of thymoma and malignant thymoma from normal thymic epithelial cells, we have examined the expression of 15 proto-oncogenes and tumor suppressor genes in seven *in vitro* epithelial cell lines established from a normal thymus (TuD1-1, TuD1-3, and TuD1-5), thymoma (TaD1-3 and TaD1-8), and malignant thymoma (MTHC-1 and MTHC-3) of rats. Northern blot analysis indicated that most of these genes examined were transcribed at similar levels. However, higher levels of transcription of epidermal growth factor receptor (EGFR) were observed in TuD1-1, TuD1-3, TuD1-5, TaD1-3, and TaD1-8 cells than in MTHC-1 and MTHC-3 cells. Conversely, four of the former five cell lines showed no TGF-β transcription while the latter two cell lines had high levels of its expression. In addition, c-*fos* proto-oncogene was highly expressed in TuD1-5 cells, which showed the fastest growth rate among the seven cell lines. These results denote that some molecular changes in the regulation of gene expression occurred in the processes of malignant transformation of thymic epithelial cells.

Key Words: Thymoma; Malignant thymoma; Expression of EGFR, TGF-β, and c-*fos*; BUF/Mna rat

INTRODUCTION

Thymomas developing in humans are classified histologically into polygonal and spindle epithelial cell types, the former being further divided into predominantly lymphocytic, mixed lymphoepithelial, and predominantly epithelial cell categories[1)]. Thymomas that show invasion to adjoining tissues and remote metastasis are diagnosed as malignant thymoma[1)]. In our laboratory, the BUF/Mna strain has been established as a high thymoma line of rats[2)]. Thymoma of predominantly lymphocytic type develops spontaneously in these rats at an incidence of nearly 100%. Crosses with the thymoma-free strain revealed that thymoma development was regulated by a dominant susceptible gene, *Tsr-1* (formerly *Tbm-1*)[3)]. *In vitro* epithelial cell lines were also established from a normal thymus, thymoma, and malignant thymoma from a ACI/NMs, (ACI/NMs × BUF/Mna)F1, or BUF/Mna rat, respectively[4,5)]. In the present study, we investigated whether these cell lines had molecular abnormality resulting in the transformation of thymic epithelia cells.

Correspondence: Dr. Mutsushi Matsuyama, Department of Pathology, Nagoya University School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466, Japan

Accepted for Publication in December 24, 1992

MATERIALS AND METHODS

Cell lines

We used seven cell lines of rat epithelial cells, derived from a normal thymus (TuD1-1, TuD1-3, and TuD1-5), thymoma (TaD1-3 and TaD1-8)[4], or malignant thymoma (MTHC-1 and MTHC-3)[5]. These cell lines were maintained in DMEM (Gibco) containing 10% fetal calf serum (FCS).

RNA extraction and Northern blot analysis

Total cellular RNA was extracted from the cell lines by a guanidinium-phenol-chloroform method[6]. The cells were harvested from ten 10-cm dishes when they became confluent. Poly(A)$^+$ RNA was isolated by a single passage through oligo(dT)-cellulose. The RNA concentrations were measured by absorbance at 260 nm. Poly(A)$^+$ RNA (4 μg/lane) was fractionated on formaldehyde/1% agarose gel and transferred to nitrocellulose filters. The filters were prehybridized, hybridized, and washed as described[7].

Plasmid and DNA probes

Plasmid DNAs of EGFR, c-*erbB-2*, c-*kit*, c-*ret*, insulin receptor, nerve growth factor receptor (NGFR, *trk*), EGF, TGF-α, TGF-β, N-*myc*, c-*myc*, c-*fos*, c-*jun*, Rb, and p53 were used as probes for Northern blot analyses. Probes of c-*fos* and c-*jun* were obtained from the Japanese Cancer Research Resources Bank.

Growth curves and doubling time

One ml of cell suspension, containing 1 to 2 $\times$ 10^5 cells, was seeded into plastic dishes (6 cm diameter) with 4 ml of DMEM containing 10% FCS. The cells were propagated at 37°C for 1, 2, 3, 4, and 5 days, respectively, changing the medium every other day, and stripped with 0.1% tripsin-0.02% EDTA. Cells were counted in a haemocytometer under a light microscope. The average of cell numbers from two dishes was calculated day by day. The same experiment was repeated twice. Doubling time (DT) was calculated, following the formula of DT = $(T_1\text{-}T_2)\log 2/\log N_1\text{-}\log N_2$. T represents time (days) and N represents cell number/ml.

Transplantability

Cells of seven cell lines were stripped from flasks with 0.1% trypsin-0.02% EDTA and centrifuged to precipitate at 1200 rpm for 3 minutes. One to 2 $\times$ 10^7 cells were injected into the subcutaneous tissue of the right axillar region of BUF/Mna-*rnu/rnu* rats. Two months later, tumors, if any, were resected, and examined histologically. Cells of these seven cell lines were cultured in DMEM containing 10% FCS for four days, and chromosome preparations were made on the 5th day. The cells were stained with 2% Giemsa's solution, and their ploidy was determined.

RESULTS

To investigate the molecular mechanisms in the development of thymoma and malignant thymoma, we compared the expression patterns of proto-oncogenes and tumor suppressor genes in the seven *in vitro* epithelial cell lines established from the normal thymus (TuD1-1, TuD1-3, and TuD1-5), thymoma (TaD1-3 and TaD1-8) and malignant thymoma (MTHC-1 and MTHC-3). Cloned genes including 6 growth factor receptor-related genes (EGFR, c-*erbB-2*,

c-*kit*, c-*ret*, insulin receptor, NGFR), 3 growth factors (EGF, TGF-α, TGF-β), 4 nuclear oncogenes (c-*myc*, N-*myc*, c-*fos*, c-*jun*) and 2 tumor suppressor genes (Rb, p53) were used as probes.

We first examined the expression of growth factor receptor-related genes. Northern blot analysis revealed that EGFR was transcribed as 10.6 kb and 5.8 kb messsages in TuD1-1, TuD1-3, TuD1-5, TaD1-3, and TaD1-8 cell lines (Fig. 1). The probe also hybridized non-specifically to 28s ribosomal RNA. On the other hand, its expression was very low in MTHC-1 and MTHC-3 cells (Fig. 1). RNAs from normal mouse liver and brain were used as positive and negative controls for EGFR expression, respectively. A 3 kb message present in the liver RNA was not observed in the cell lines. Transcritption of other genes (c-*erbB-2*, c-*kit*, c-*ret*, insulin receptor, NGFR) was undetectable in all of the cell lines (data not shown).

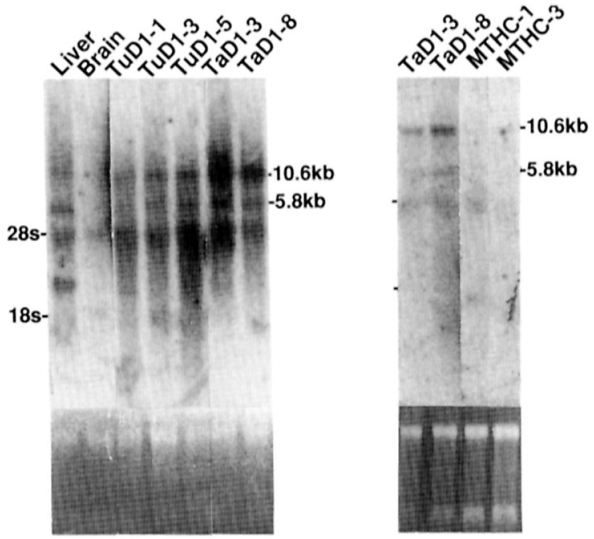

Fig. 1. Northern blot analyses of RNAs from TuD1-1, TuD1-3, TuD1-5, TaD1-3, TaD1-8, MTHC-1, and MTHC-3 cell lines. Top: Poly $(A)^+$ RNA (4 μg) was analyzed by Northern blot hybridization with EGFR probe. Liver and brain tissues were used as positive and negative controls for EGFR expression, respectively.
Bottom: RNAs were stained with ethidium bromide.

In contrast to the expression pattern of EGFR, TGF-β, one of the growth factors, was transcribed at high levels in MTHC-1 and MTHC-3 cells but not in TuD1-1, TuD1-3, TaD1-3, and TaD1-8 cells (Fig. 2). It was slightly expressed in TuD1-5 cells. Transcrition of TGF-α was observed at higher levels in TuD1-5, TaD1-8, and MTHC-3 cells than in the other four cell lines (Fig. 2) and EGF showed no expression in any of the cell lines (data not shown).

The c-*myc* gene was transcribed as a 2.4 kb mRNA in the seven cell lines although there were some differences in the expression levels (Fig. 3). The c-*myc* probe non-specifically hybridized to 28s ribosomal RNA. In contrast, no transcription of N-*myc* and c-*jun* genes was detected in them (data not shown). In the case of c-*fos* gene, an exceptionally high level of its expression was observed in TuD1-5 cells (Fig. 3). It is interesting to note that TuD1-5 cells showed the fastest growth rate among the seven cell lines as described below. The c-*fos* gene was also expressed at variable levels in the other six lines (Fig. 3).

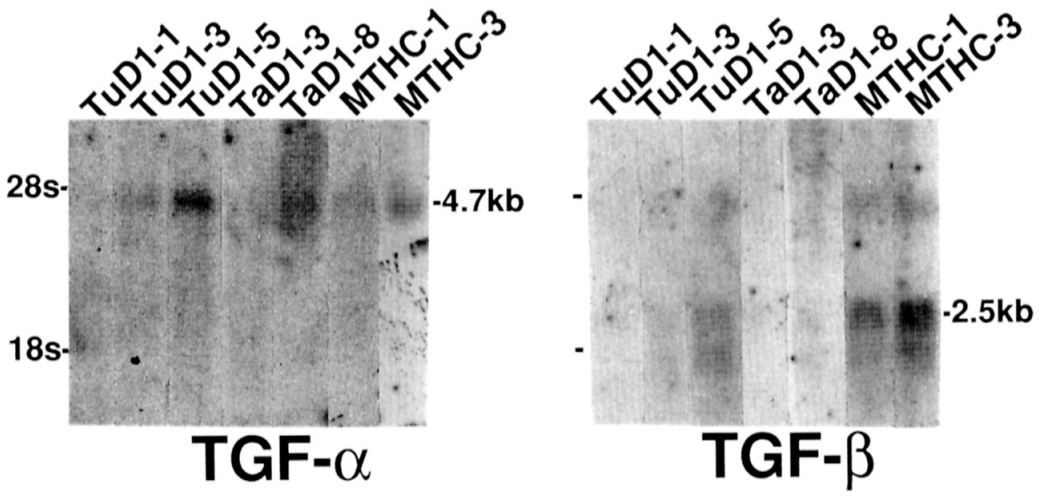

Fig. 2. Northern blot analyses of RNAs from TuD1-1, TuD1-3, TuD1-5, TaD1-3, TaD1-8, MTHC-1, and MTHC-3 cell lines, with TGF-α and TGF-β probes.

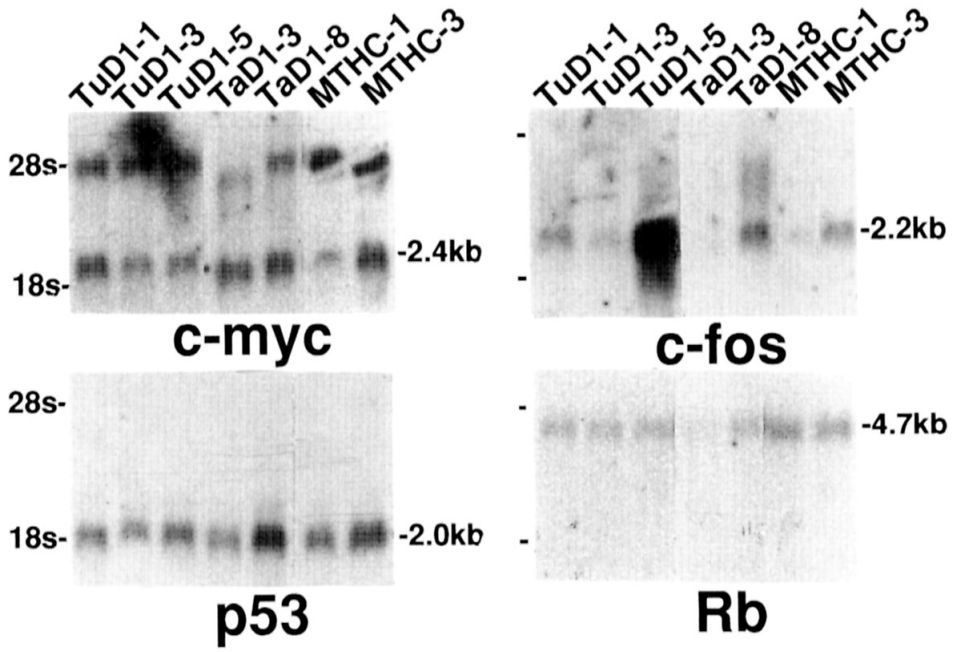

Fig. 3. Northern blot analyses of RNAs from TuD1-1, TuD1-3, TuD1-5, TaD1-3, TaD1-8, MTHC-1, and MTHC-3 cell lines, with c-*myc*, c-*fos*, Rb, and p53 probes.

The expression of the Rb gene, one of the tumor suppressor genes, was detected as a weak band of 4.7 kb mRNA in the seven cell lines (Fig. 3). The p53 gene showed relatively high levels of expression in them (Fig. 3).

There were slight differences in the doubling time among the seven lines, demonstrating that TuD1-5 cells have the fastest growth rate (Table 1). Karyotype analysis indicated that TuD1-1 and TuD1-5 cells were diploid and the other five cells were hypotetraploid (Table 1). When 1 to 2×10^7 cells were injected into the subcutaneous tissue of nude rats, TuD1-3, TuD1-5, TaD1-8, MTHC-1, and MTHC-3 cells but not TuD1-1 cells showed tumorigenicity (Table 1). TuD1-5 and TaD1-8 cells formed cystic tumors, lined by epithelial cells, and TuD1-3, MTHC-1, and MTHC-3 produced solid tumors.

Table 1. Doubling Time, Karyotype, and Transplantability of Cell Lines Derived from the Normal Thymus, Thymoma, and Malignant Thymoma.

Cell Line	Doubling time	Karyotype	Transplantability[a)]
TuD1-1	23.0	diploid	− (0/1)
TuD1-3	19.4	hypotetraploid	+ (1/1)
TuD1-5	16.2	diploid	+ (5/5[b)])
TaD1-3	24.0	hypotetraploid	NT[c)]
TaD1-8	26.5	hypotetraploid	+ (1/1[b)])
MTHC-1	20.3	hypotetraploid	+ (5/5)
MTHC-3	27.1	hypotetraploid	+ (5/5)

a) Transplantability was assessed by subcutaneous injection of more than 1×10^7 cells into the subcutaneous tissue of the right axillar region of BUF/Mna-*rnu/rnu* rats. The number of positive rats/total number of injected rats is shown in parentheses.
b) Cells proliferated and formed large cysts lined by squamous epithelial cells, associated with papillary projections into the lumen.
c) NT: Not tested.

DISCUSSION

The results reported here indicate that MTHC-1 and MTHC-3, derived from the malignant thymoma of the BUF/Mna rat, showed increased expression of TGF-β and decreased expression of EGFR when compared with the cell lines derived from the normal thymus and thymoma. Since all of the cell lines used show similar growth behavior *in vitro* and the three cell lines, TuD1-3, TuD1-5, TaD1-8, derived from normal thymus and thymoma, as well as malignant thymoma cell lines showed tumorigenicity in nude rats, the changes of expression of TGF-β and EGFR may not influence the grade of malignancy in *in vitro* of the cell lines. Rather, it is conceivable that these changes have already occurred in original malignant thymoma of a rat. These results might correspond to the fact that *in vitro* high-metastatic variants derived from Lewis lung carcinoma of the mouse expressed TGF-β at higher levels than low-metastatic variants, whereas similar expressions were found in cell cycle-related genes (vimentin, calcyclin, c-*myc*, and p53) and oncogenes (Ki-*ras*, Ha-*ras*, c-*sis*, c-*src*, c-*fes*, and c-*erbB*)[8)]. Human poorly differentiated adenocarcinoma also showed an excessive expression of TGF-β when compared with well-differentiated adenocarcinoma[9)]. It is, therefore, conceivable that the changes of expression pattern of EGFR and TGF-β may reflect the grade of malignancy in the rat thymomas. These findings indicate that some alterations in the regulation of gene expression occurred in the processes of malignant transformation of thymic epithelial cells.

An additional point made by the present study is that TuD1-5, derived from the normal thymus, showed an intensive expression of c-*fos*. Southern blot analysis showed that amplification

of c-*fos* DNA was not found in TuD1-5 cells (data not shown). The high expression of c-*fos* is well fitted to the biological property of the TuD1-5 cells, whose growth rate was the fastest among the seven cell lines. The mechanism of the regulation of the c-*fos* expression in TuD1-5 should be studied in the future.

ACKNOWLEDGMENTS

We thank Professor E. Tahara, Hiroshima University School of Medicine, for providing us with cDNAs of EGFR and TGF-β. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan and from the Ministry of Health and Welfare, Japan.

REFERENCES

1) Lewis, J. E., Wick, M. R., Scheithauer, B. W., Bernatz, P. E. and Taylor, W.: Thymoma. A clinicopathologic review. *Cancer,* 60, 2727-2743 (1987).
2) Matsuyama, M. and Nagayo, T.: Development of thymoma and myasthenia in Buffalo/Mna rats. *Proc. Jpn. Cancer Assoc.,* 36th Ann. Meet., 30 (1997).
3) Matsuyama, M., Yamada, C. and Hiai, H.: A single dominant susceptible gene determines spontaneous development of thymoma in BUF/Mna rat. *Jpn. J. Cancer Res. (Gann),* 77, 1066-1068 (1986).
4) Masuda, A., Ohtsuka, K. and Matsuyama, M.: Establishment of functional epithelial cell lines from a rat thymoma and a rat thymus. *In Vitro Cell. Dev. Biol.,* 26, 713-721 (1990).
5) Lu, J., Sakai, Y., Wajjawalku, W., Isobe, K., Saito, M., Amo, H., Kojima, A., Utsumi, K. R., Takahashi, M., Hiai, H. and Matsuyama, M.: Establishment of transplantable tumor lines and in vitro cell lines of malignant thymoma developed in a BUF/Mna rat. *Jpn. J. Cancer Res.,* 83, 618-624 (1992).
6) Chomczynski, P. and Sacchi, N.: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* 162, 156-159 (1987).
7) Maniatis, T., Fritsch, E. F. and Sambrook, J.: (1982) Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Lab., New York.
8) Perrotti, D., Cimino, L., Ferrari, S. and Sacchi, A.: Differential expression of transforming growth factor-β1 gene in 3LL metastatic variants. *Cancer Res.,* 51, 5491-5494 (1991).
9) Tahara, E.: Occurrence, growth and progression of human gastric cancer: a molecular pathological approach. *Tr. Soc. Pathol. Jpn.,* 81, 21-49 (1992) (in Japanese).