BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 658–664 (1997)
ARTICLE NO. RC977262

Involvement of *Sonic hedgehog* in the Cell Growth of LK-2 Cells, Human Lung Squamous Carcinoma Cells

Eriko Fujita,* Yoriko Khoroku,* Koko Urase,* Toshifumi Tsukahara,† Mariko Y. Momoi,‡ Hiromichi Kumagai,§ Tamiko Takemura,¶ Toshio Kuroki,‖ and Takashi Momoi*,[1]

*Division of Development and Differentiation and †Department of Neuromuscular Research, National Institute of Neuroscience, NCNP, Ogawahigashi-machi, Kodaira, Tokyo 187, Japan; ‡Department of Pediatrics, Jichi Medical School, Minamikawachi, Kawachi-gun, Yakushiji, Tochigi 329-04, Japan; §Asahi Glass Co., 1150 Hasawa-cho, Kanagawa-ku Yokohama, Kanagawa 221, Japan; ¶Department of Pathology, Japanese Red Cross Medical Center, Hiroh, Shibuya-ku, Tokyo 150, Japan; and ‖Molecular Oncology Research Center, Shouwa University, Hatanodai, Shinagawa-ku, Tokyo 142, Japan

Received July 30, 1997

Mutation of the *Patched* gene has been detected in human inherited basal cell nevus syndrome (BCNS) and sporadic basal cell carcinomas (BCC), suggesting a strong relation between a Sonic hedgehog-Patched signal and cell proliferation. In the present study, we demonstrate that *Sonic hedgehog* is expressed in human lung squamous carcinoma (LK-2 and EBC-1) and some adenocarcinoma cell lines. The expression of *Sonic hedgehog* is also detected in the human lung squamous carcinoma tissues, but not in the normal lung tissue of the same patient. The N-terminal region of Sonic hedgehog stimulates the incorporation of BrdU into LK-2 cells and stimulates their cell growth, while anti-Shh-N inhibits their cell growth. These results suggest that a Sonic hedgehog signal is involved in the cell growth of LK-2 cells.

Sonic hedgehog (*Shh*), which is a vertebrate homologue of *hedgehog* (*hh*), a segment polarity gene, is specifically expressed in the notochord and floor plate in early developmental stages, and zone polarizing activity (ZPA) in the limb buds and epithelium of lung and digestive organs in later developmental stages (1-5). Very recently, a mutation was detected in human *Shh* in the patients of holoprosencephaly (6). Targeted gene disruption of *Shh* in the mouse shows abnormal development of the brain, spinal cord, axial skeleton, and the limbs (7). Thus *Shh* is involved in the various morphogeneses of vertebrates.

The *Patched* (*PTC*) gene, identified initially as the *Drosophila* segment polarity gene, encodes a transmembrane protein (8-12). Recently, it has been shown that PTC has an ability to bind with Shh, possibly as its receptor (13,14). Patched constitutively represses downstream targets and the hh signal relieves *ptc* repression, thereby inducing transcription of the downstream genes in *Drosophila* (15-17). The mouse mammary oncogene *Wnt1* and the human glioblastoma oncogene *GLI* are homologues of the *Drosophila* segment polarity genes, *wingless* and *Cubitus interruptus*, respectively, which are components of the Hh-PTC signal transduction pathway (18,19).

Recently, the relationship between gene pathways of Shh-PTC controlling embryonic development and those involved in regulating cell proliferation has become a focus of study. Shh (~45kDa) itself catalyzes the intramolecular cleavage that generates Shh-N (~20kDa) and Shh-C (~29kDa) (20). Shh-N, which is sufficient to induce the duplication of digits (21) and motor neurons (22,23), has an mitogenic effect on the presomitic mesoderm cells *in vitro* (24). Overexpression of Shh induces a mitogenic effect on mouse lung mesenchymal cells and skin keratinocytes *in vivo* (25,26).

The basal cell nevus syndrome (BCNS) is characterized by developmental abnormalities and by the postnatal occurrence of cancers, especially basal carcinomas (BCCs), the most common human cancer. Very recently, a heritable mutation in patients with BCNS and a somatic mutation in sporadic BCC were identified in human *PTC* gene (27,28). Human *PTC* is a strong candidate gene for BCNS. If so, it is very likely that *PTC* plays a role as a tumor suppresser. Moreover, a mutation is also found in *Shh* gene of the patients with BCNS (26). These results suggest that Shh-PTC pathway is closely associated with cell proliferation in several types of adult cells.

In the present study, we focus on the relationship between the Shh-PTC pathway and the cell growth of human lung squamous carcinoma (LK-2) cells. We show that Shh-N stimulates the incorporation of BrdU

0006-291X/97 $25.00

into LK-2 cells and their cell growth, while anti-Shh-N prevents the cell growth of LK-2 cells.

MATERIALS AND METHODS

Cell culture. Human lung cancer lines, squamous carcinoma cells (EBC-1 and LK-2), adenocarcinoma cells (RERF-LCOK, VMRC-LCD, ABC-1), lung small cell carcinoma cells (RERF-LCMA, SBC-1, and SBC-2), human squamous carcinoma cells of mouth (NA, HSC-2), human epidermoid carcinoma cells (A431) were obtained from the Japanese Cancer Research Bank (Tokyo Japan). Human neuroblastoma cells (NB-1, IMR-32), human leukemia cells (U937 and Jurkat cells), and human teratocarcinoma cells (NT-2) were obtained from ATCC. These cells were cultured in α-minimum essential medium containing 10% fetal calf serum. In experiments to examine the effect of Shh on the cell growth,

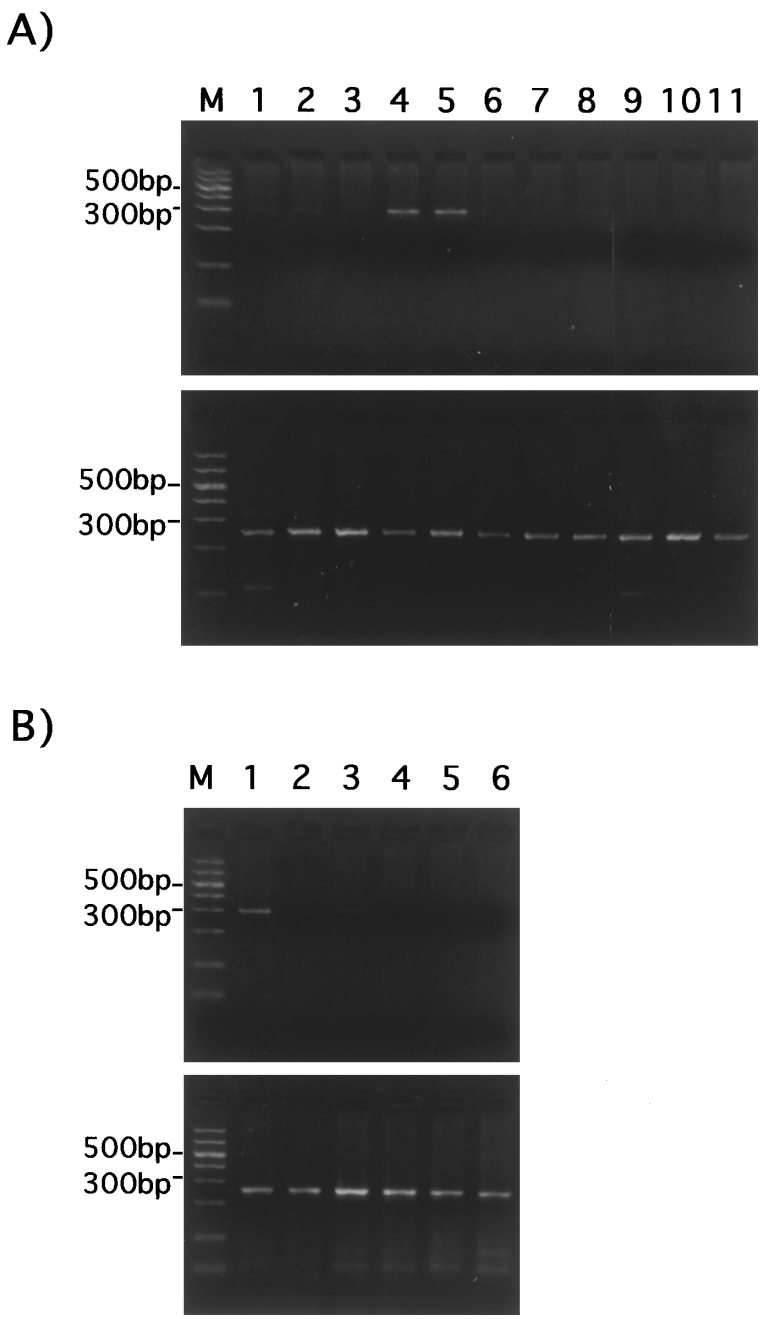

FIG. 1. RT-PCR of *Shh* and *PTC* of the human cancer cells. (A) Human lung cancer cells and squamous carcinoma cells. Lane 1, RERF-LCOK; lane 2, VMRC-LCD; lane 3, ABC-1; lane 4, EBC-1; lane 5, LK-2; lane 6, RERF-LCMA; lane 7, SBC-1; lane 8, SBC-2; lane 9, A431; lane 10, NA; lane 11, HSC-2. (B) Other human cancer cells. lane 1, LK-2; lane 2, NT-2; lane 3, NB-1; lane 4, IMR-32; lane 5, U937; lane 6, Jurkat cells. Upper panel is *Shh* and lower panel is *PTC*.

LK-2 cells were cultured with histag-Shh-N or anti-Shh-N at various concentration in the fetal calf serum free culture medium.

Incorporation of BrdU. Human lung carcinoma cells were inoculated at 1.1×10^5 cells/cm^2 and incubated in the medium containing fetal calf serum for 6 h, followed with the incubation with serum free medium for 8 h, and histag-Shh-N was added. After incubation for 12 h, BrdU (3 μg/ml) was added in the culture medium and incubated for 1h. Then cells were washed with PBS and subjected to the detection of the incorporation of BrdU according to the manufacture protocol by immunostaining using anti-BrdU.

Preparation of His-tag fusion protein of Shh-N. Rat Shh-N was generated by polymerase chain reaction and subcloned into pET15b expression vector (Novagen). Restorations of reading frames were confirmed by DNA sequencing. The his-tag fusion protein of Shh-N was produced according to the manual. His-tag fusion protein was purified by Nickel-affinity column chromatography.

RT-PCR (reverse transcript-polymerase chain reaction. The expression of *Shh* and *PTC* in human cancer cells was examined by RT-PCR using 5′-CCAATTACAACCCCGACATC-3′ and 5′-TCGTAGTACACCCAGTCGAA-3′ for *Shh.* and 5′-TATTACACCTTTGGACTGCTTCTGGGAA-3′ and 5′-TTGAATTTTTGTTGGGGGCTGTGGCGGG-3′ for *PTC.* Total RNA was prepared from human cancer cells according to the guanidium thiocyanate method (29). The total RNA (1 μg) was subjected to RT-PCR as described in the manual from Perkin-Elmer, 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 1 min, 30 cycles at 60°C for 2 min, and 1 cycle 60°C for 7 min. The PCR products were subjected to 3% Nusieve gel (Takara, Kyoto) electrophoresis.

Immunoblot analysis. The antiserum (anti-Shh-N serum) against RLAVEAGFDWVYYESC, synthetic peptide of Shh-N, was prepared (30). After anti-Shh-N serum was purified by peptide affinity column chromatography, reactivities of this antiserum against fusion protein of rat Shh-N were examined by immunoblot analysis. Recombinant His-tagged proteins were solubilized with 6M urea from cells. Human Shh was extracted from LK-2 cells with 50 mM Tris-HCl (pH7.5) containing 150 mM NaCl and 1% Triton X-100. After centrifugation, aliquots (each containing 50 μg protein) were subjected into SDS polyacrylamide (12%) electrophoresis and immunoblot analysis. The proteins in the gels were electrophoretically transferred to nitrocellulose filter, and the filters were incubated overnight with anti-Shh-N at 4°C and then incubated with alkaliphosphatase-conjugated goat anti-rabbit immunoglobulin (Promega, Madison, WI). Immuno-reactivities were visualized by incubating the filter in the reaction buffer (100 mM Tris-HCl (pH9.5), 100 mM NaCl, 5 mM $MgCl_2$, 40 mM 5-bromo-4-chloro-3-indolylphosphatte, and 80 mM nitro blue tetrazolium salt) at room temperature.

Immunostaining. The localization of Shh in lung squamous carcinoma cell lines, and the distribution of Shh in lung squamous tissues were examined by immunostaining using anti-Shh-N, which specifically react with lung epithelium of mouse embryos (30). After carcinoma cells and cancer tissue sections were fixed with 4% paraformaldehyde, they were incubated with anti-Shh-N antiserum at 4°C for 3 days, and the immunoreactivities on the cells and the each section were detected by a peroxidase-conjugated avidin-biotin kit (Vector Laboratories, CA).

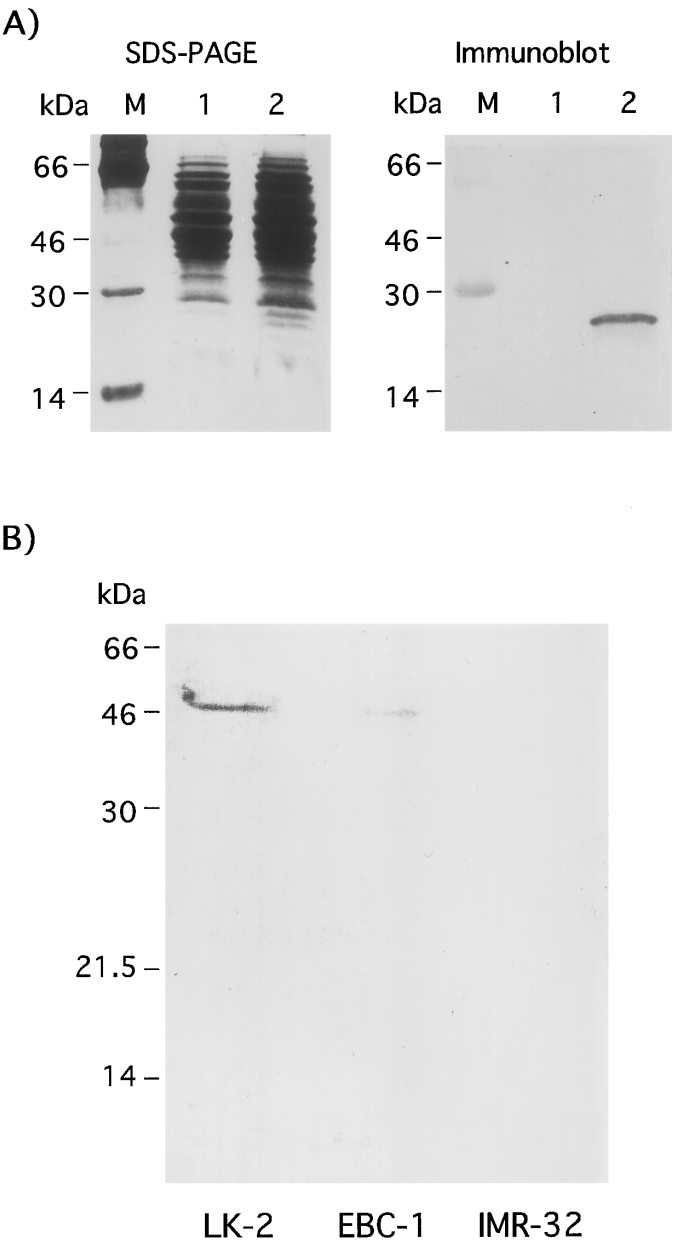

FIG. 2. Immunoblot analysis of Shh expressed in LK-2 cells by anti-Shh-N.(A) Reactivity of anti-Shh antiserum against histag-Shh-N fusion protein. Lane 1 correspond to cells transformed with pET15b vector alone, lane 2 to cells transformed with rat Shh-N conjugated with pET15b vector. (B) Immunoblot analysis of Shh expressed in human cancer cells. LK-2 cells (lane 1), EBC-1 cells (lane 2), and IMR-32 (lane 3).

RESULTS

Shh was expressed in the human lung squamous carcinoma (LK-2 and EBC-1) cells, weakly expressed in some of lung adenocarcinoma (RERF-LCOK, VMRC-LCD) and neuroblastoma cells (NB-1), but not in lung small cell carcinoma cells (RERF-LCMA, SBC-1, and SBC-2), leukemia cells (U937 and Jurkat cells), IMR-32 (neuroblastoma cells), NT-2 (teratocarcinoma) or mouth squamous carcinoma (HSC and NA) and A431 (epidermoid carcinoma cells) derived from ectoderm (Figure 1A). In contrast with *Shh*, *PTC* (250-bp) was detected by RT-PCR in all the cell lines examined (Figure 1B). The nucleotide sequences of PCR products of *Shh* and *PTC* of LK-2 cells was completely identical to those of *Shh* and *PTC* previously reported, respectively (10, 12, 31).

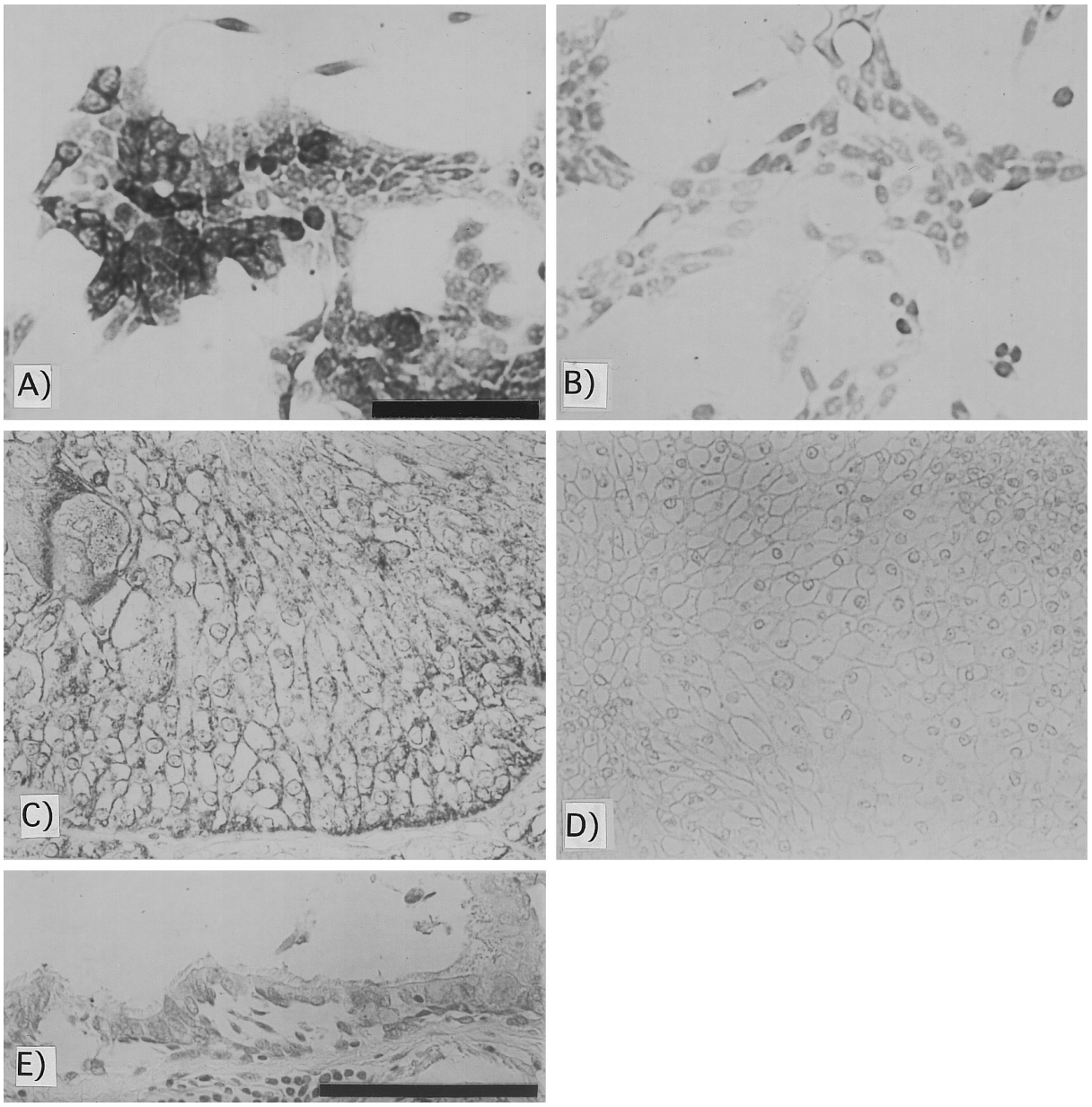

FIG. 3. Immunostaining of Shh of human lung squamous carcinoma cells and tissues. LK-2 cells (A and B), lung squamous carcinoma tissues from 58-year-old man (C and D) and normal lung tissues of the same patient with lung squamous carcinoma (E). Immunostaining with anti-Shh-N (A, C, and E) and immunostaining with normal rabbit serum (B and D). Bars indicate 100 μm.

Anti-Shh reacted with a 20 kDa band of histag-Shh-N fusion protein (Figure 2A). Human Shh (47 kDa) was detected in LK-2 cells and EBC-1 cells by immunoblot analysis using anti-Shh-N, but not detected in IMR-32 cells (Figure 2B). However, Shh-N (20 kDa), an endogenous cleavage fragment of Shh, was not detected in LK-2 cells and EBC-1 cells probably because of the small amount. Localization of Shh protein in LK-2 cells was examined by immunostaining using anti-Shh-N. Shh was mainly localized in the plasma membrane and partly in cytosol in LK-2 cells (Figure 3A), while Shh was negative in IMR-32 cells (unpublished observation). Shh was also positive in the three lung squamous carcinoma tissues (Figure 3C), while Shh was negative in the epithelium of normal lung tissues of the same patients (Figure 3E).

In the absence of fetal calf serum, Shh-N (1-1000 ng/ml) stimulated the incorporation of BrdU into LK-2 cells (Figure 4A). The stimulation of the BrdU-incorporation by Shh-N was shown in the lung squamous carcinoma cells, LK-2 and EBC cells, but not in the adenocarcinoma cells, RERF-LCOK and ABC-1 cells, and in the lung small cell carcinoma, RERF-LCMA and SBC-2 cells (Figure 4B).

Some population of LK-2 cells had an ability to maintain the cell growth in the absence of fetal calf serum autonomously. Shh-N (10 ng/ml) stimulated the cell growth of LK-2 cells, but not that of ABC-1 cells, 2-3 times more than unstimulated cells by 6-day incubation (Figure 5A and B). To examine the effect of Shh expressed in LK-2 cells on their cell growth, LK-2 cells were incubated in the presence or absence of anti-Shh-N. The cell growth of LK-2 cells was concentration-dependently inhibited by anti-Shh-N, but not by normal rabbit immunoglobulin (Figure 5C). Thus LK-2 cells failed to grow in the presence of anti-Shh-N (100 ng/ml).

DISCUSSION

Shh is intensely expressed in the mouse lung epithelium during branching morphogenesis, and decreases during development (25,30,32). In adult mouse lung tissues, the expression of *Shh* is faintly detected. In contrast with *Shh*, *PTC* is expressed in the mesenchymal cells located near the epithelium of the distal tips of bronchial tubes, in which *Shh* is intensely expressed. Overexpression of *Shh* in lung epithelium has a mitogenic effect on the lung mesenchymal cells *in vivo* (25). Thus Shh secreted from lung epithelium has a growth effect on the mesenchymal cells by the Shh-PTC pathway by paracrine mechanisms.

As shown in Figure 3C, Shh was positive in the squamous carcinoma, but it was not detected in normal lung tissues of the same patient. Shh seems to disappear in the adult human lung epithelial cells during aging, but it seems to appear in the lung squamous carcinoma cells. Thus far, *Shh* has been shown to be positive in all human lung squamous carcinoma cells (LK-2 and EBC-1) (Figure 1) and human lung

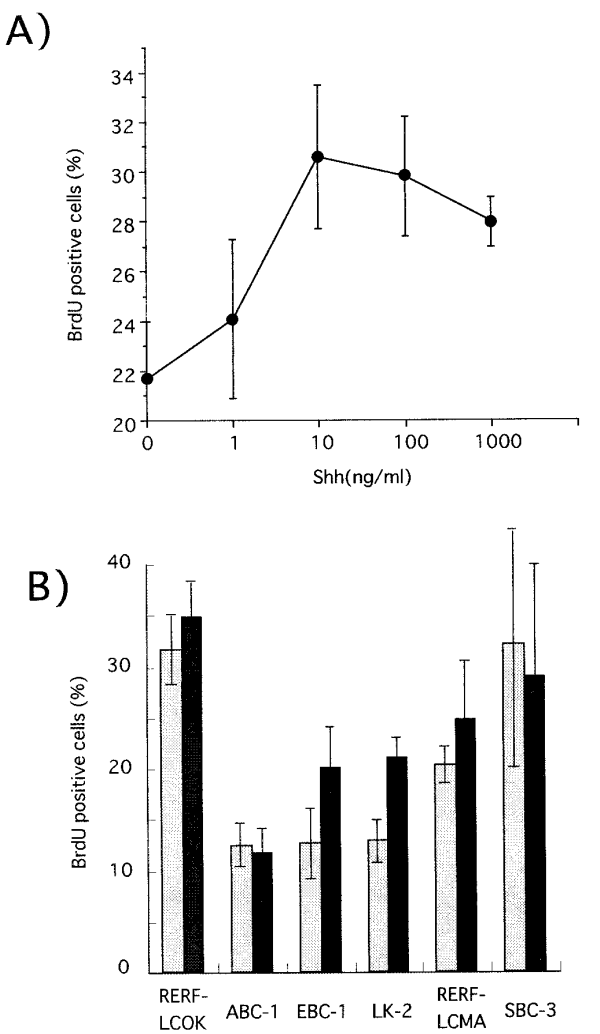

FIG. 4. Stimulation of BrdU-incorporation by Shh-N.(A) Dose-dependent incorporation of BrdU into LK-2 cells by Shh-N. (B) BrdU-incorporation into various lung carcinoma cells in the presence (black bars) or absence (gray bars) of Shh-N. Values are average of number of BrdU-positive cells of three experiments.

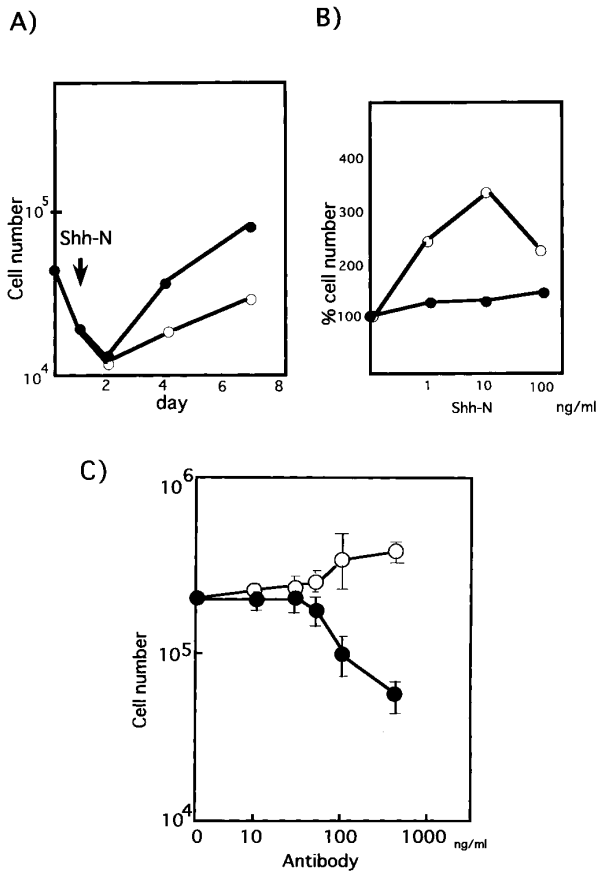

FIG. 5. Effect of Shh-N and anti-Shh-N on the cell growth of LK-2 cells. (A) The cell growth of LK-2 cells in the presence (closed circles) or absence (open circles) of Shh-N. Shh-N (10 ng/ml) was added at day one. (B) Dose-dependent effect of Shh-N on the cell growth of LK-2 cells (open circles) and ABC-1 cells (closed circles). (C) Effects of anti-Shh-N on the cell growth of LK-2 cells. LK-2 cells were incubated in the presence or absence of anti-Shh-N for 6 days. Cell growth of LK-2 cells in the presence of anti-Shh-N antibody (closed circles) or normal rabbit immunoglobulin (open circles).

squamous carcinoma tissues (n=3) we examined. In addition, other squamous carcinomas (T.T. and T.Tn.) derived from endoderm epithelial cells of the esophagus were Shh-positive (unpublished observation), while squamous carcinoma cells derived from ectoderm epithelial cells such as A431, NA, and HSC-2 were Shh-negative (Figure 1). Shh was weakly positive in the some of the lung adenocarcinoma, RERF-LCOK and VMRC-LCD (Figure 1), stomach adenocarcinoma (MKN-21), and colon adenocarcinoma (SW480) (unpublished observation). Thus the expression of *Shh* seems to be restricted to the squamous carcinoma and some of the adenocarcinoma derived from endoderm epithelial cells, which express Shh in the embryonic stage (5).

In contrast to *Shh*, *PTC* was expressed not only in the lung squamous carcinoma but also in all other cancer cells examined (Figure 1). However, if PTC is only a receptor for Shh, it is not clear why stimulation of BrdU-incorporation induced by Shh-N is restricted to lung squamous carcinoma, LK-2 and EBC-1, cells (Figure 4). One of the possibility is that there may be a mutation in *PTC* gene of lung squamous carcinoma cells as shown in the patient with BCNS or sporadic BCC (27,28). The other possibility is that the Shh-PTC signal is different in lung squamous carcinoma and other type of lung carcinoma cells. Recently, *Smoothened* (*Smo*) has been postulated to be one of the positive signaling units interacting with PTC (13,33). The expression of Smo or interaction between Smo and PTC may be different in lung squamous carcinoma and other type of lung carcinoma.

Shh-N stimulated the cell growth of LK-2 cells, while anti-Shh-N inhibited the cell growth of LK-2 cells (Figure 5). Thus Shh of LK-2 cells seems to stimulate their own cell growth through interaction with PTC by an autocrine mechanism. LK-2 cells are useful for the study on the Shh-PTC signal involved in the cell proliferation.

ACKNOWLEDGMENTS

This work was supported in part by Research Grants 5A-1 for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan and by Grants-in-Aid for Scientific Research on Priority Areas (No 05277102 and 08265273) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Echelard, Y., Epstein, D. J., St-Jacqes, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993) *Cell* **75,** 1411–1430.
2. Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993) *Cell* **75,** 1401–1416.
3. Krauss, S., Concordet, J. P., and Ingham, P. W. (1993) *Cell* **75,** 1431–1444.
4. Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., RuiziAltaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessel, T. M., and Dodd, J. (1994) *Cell* **76,** 761–775.
5. Bitgood, M. J., and McMahon, A. P. (1995) *Dev. Biol.* **172,** 126–138.
6. Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P. et al. (1996) *Nature genetic* **14,** 357–360.
7. Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996) *Nature* **383,** 407–413.
8. Hooper, J. E., and Scott, M. P. (1989) *Cell* **59,** 751–765.
9. Nakano, Y., Guerrero, I., Hidelgo, A., Taylor, A., Whittle, J. R. S., and Ingham, P. W. A. (1989) *Nature* **341,** 508–513.
10. Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., and Scott, M. P. (1996) *Gene & Dev.* **10,** 301–312.
11. Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V., and Tabin, C. J. (1996) *Development* **122,** 1225–1233.
12. Hahn, H., Christiansen, J., Wicking, C., Zaphiropoulos, P. G., Chidambaram, A., Gerrard, B., Vorechovsky, I., Bale, A. E., Toftgard, R., Dean, M., and Wainwright, B. (1996) *J. Biol. Chem.* **271,** 12125–12128.
13. Stone, D. M., Hynes, M., Armanini, M., Swanson, T. D., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F., and Rosentthaal, A. (1996) *Nature* **384,** 129–134.
14. Marigo, V., Davey, R. A., Zuo, Yi., Cunningham, J. M., and Tabin, C. J. (1996) *Development* **384,** 176–179.
15. Ingham, P. W., Taylor, A. M., and Nakano, Y. (1991) *Nature* **353,** 184–187.
16. Hidalgo, A., and Ingham, P. (1990) *Development* **110,** 291–301.
17. Capdevila, J., and Guerrero, I. (1994) *EMBO J.* **13,** 4459–4468.
18. Rijsewik, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987) *Cell* **50,** 649–657.
19. Hui, C., Slusarski, D., Platt, K. A., Holmgren, R., and Joyner, A. L. (1994) *Dev. Biol.* **162,** 402–413.
20. Lee, J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I., and Beachy, P. A. (1994) *Science* **266,** 1528–1537.
21. Lopez-Martinez, A., Chang, D. T., Chiang, C., Porter, J. A., Ros, M. A., Simandl, B. K., Beachy, P. A., and Fallon, J. F. (1995) *Curr. Biol.* **5,** 791–796.
22. Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995) *Cell* **81,** 445–455.
23. Marti, F., Bumcrot, D. A., Takeda, R., and McMahon, A. P. (1995) *Nature* **375,** 322–324.
24. Fan, C.-M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A., and Tessier-Lavigne, M. (1995) *Cell* **81,** 457–465.
25. Bullusci, S., Furuta, Y., Ruth, M. G., Henderson, R., Winnier, G., and Hogan, B. L. M. (1997) *Development* **124,** 53–63.
26. Oro, A. E., Higgins, K. H., Hu, Z., Bonifas, J. M., Epstein, I. E. H., Scott, M. D. (1997) *Science* **276,** 817–821.
27. Hahn, H., Wicking, C., Zaphiropoooulos, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Syth, I., Pressman, C., Leffell, D. L., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Trench, G. C., Wainwright, B., and Baale, A. E. (1996) *Cell* **85,** 841–851.
28. Johnson, R., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, Jr. E. H., and Scott, M. P. (1996) *Science* **272,** 1668–1671.
29. Sambrook, D., Frritch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. Urase, K., Mukasa, T., Igarashi, H., Ishii, Y., Yasugi, S., Y-Momoi, M., and Momoi, T. (1996) *Biochem. Biophys. Res. Commun.* **210,** 231–237.

31. Marigo, V., Roberts, D. J., Lee, S. M., Tsukurov, O., Levi, T., Gastier, J. M., Epstein, D. J., Gilbert, D. J., Copeland, N. G., Seidman, C. E., and Tabin, D. J. (1995) *Genomics* **28,** 44–51.
32. Bellusci, S., Henderson, R., Winnier, G., Oikawa, T., and Hogan, B. L. M. (1996) *Development* **122,** 1693–1702.
33. Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J. E. (1996) *Cell* **86,** 221–232.