Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3636–3641, April 1996
Medical Sciences

Amplification of *AKT2* in human pancreatic cancer cells and inhibition of *AKT2* expression and tumorigenicity by antisense RNA

JIN QUAN CHENG*, BRUCE RUGGERI†, WALTER M. KLEIN*, GONOSUKE SONODA*, DEBORAH A. ALTOMARE*, DENNIS K. WATSON‡, AND JOSEPH R. TESTA*§

*Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111; †Department of Pathology, Medical College of Pennsylvania, Philadelphia, PA 19129; and ‡Center for Molecular and Structural Biology, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC 29425

Communicated by Alfred G. Knudson, Fox Chase Cancer Center, Philadelphia, PA, December 20, 1995 (received for review April 15, 1995)

ABSTRACT **We previously demonstrated that the putative oncogene *AKT2* is amplified and overexpressed in some human ovarian carcinomas. We have now identified amplification of *AKT2* in ≈10% of pancreatic carcinomas (2 of 18 cell lines and 1 of 10 primary tumor specimens). The two cell lines with altered *AKT2* (PANC1 and ASPC1) exhibited 30-fold and 50-fold amplification of *AKT2*, respectively, and highly elevated levels of *AKT2* RNA and protein. PANC1 cells were transfected with antisense *AKT2*, and several clones were established after G418 selection. The expression of AKT2 protein in these clones was greatly decreased by the antisense RNA. Furthermore, tumorigenicity in nude mice was markedly reduced in PANC1 cells expressing antisense *AKT2* RNA. To examine further whether overexpression of *AKT2* plays a significant role in pancreatic tumorigenesis, PANC1 cells and ASPC1 cells, as well as pancreatic carcinoma cells that do not overexpress *AKT2* (COLO 357), were transfected with antisense *AKT2*, and their growth and invasiveness were characterized by a rat tracheal xenotransplant assay. ASPC1 and PANC1 cells expressing antisense *AKT2* RNA remained confined to the tracheal lumen, whereas the respective parental cells invaded the tracheal wall. In contrast, no difference was seen in the growth pattern between parental and antisense-treated COLO 357 cells. These data suggest that overexpression of *AKT2* contributes to the malignant phenotype of a subset of human ductal pancreatic cancers.**

AKT2 was identified as one of the human homologs of the v-*akt* oncogene (1, 2). *AKT2* encodes a protein-serine/threonine kinase containing a Src homology 2-like region (1), now known to be contained within a pleckstrin homology domain, a region found in a diverse group of signaling proteins (3, 4). Recently, c-Akt and human AKT2 were identified as downstream targets of phosphatidylinositol-3-OH kinase (5, 6). c-Akt and AKT2 can be activated by stimuli such as platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, and insulin through phosphatidylinositol-3-OH kinase, suggesting that members of the Akt family are important signal mediators that may contribute to the control of cell proliferation and malignant transformation (5–7).

We previously demonstrated that *AKT2* is amplified and overexpressed in some human ovarian cancers, and such alterations of *AKT2* may be associated with a poor prognosis (1, 8). In a screen of other tumor types for alterations of *AKT2*, we have now identified amplification of this gene in 10% of primary ductal pancreatic tumor specimens. In addition, amplification and greatly enhanced expression of *AKT2* were observed in two pancreatic cancer cell lines.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Antisense RNA, antisense oligonucleotides, and ribozymes that are specific in targeting individual oncogenes have been used to successfully suppress tumorigenesis in different human cancer cell lines by reversing the transformed phenotype (9–16). Such promising results suggest that antisense technology could be a potentially important approach for cancer gene therapy. To determine whether overexpression of *AKT2* contributes to the malignant cell phenotype, pancreatic tumor cells that have amplified and overexpressed *AKT2* were transfected with an antisense *AKT2* construct. Tumorigenicity in nude mice and invasiveness as ascertained by a rat tracheal xenotransplant assay were significantly reduced in tumor cell lines with altered *AKT2* that were transfected with antisense RNA.

MATERIALS AND METHODS

Tumor Specimens and Cell Lines. Eighteen human pancreatic carcinoma cell lines were either obtained from the American Type Culture Collection or established from surgically explanted primary ductal pancreatic carcinomas. All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum. Primary pancreatic carcinoma specimens were obtained through the Cooperative Human Tissue Network from patients who had not received radiation and/or chemotherapy prior to surgical resection of their tumor. Epithelial cellularity of tumor samples was a minimum of 30%.

Southern Blot, Northern Blot, and Reverse Transcriptase–PCR Analyses. DNA from cell lines and tumor specimens was isolated following our standard method (1). Total cellular RNA was extracted by a one-step guanidinium isothiocyanate-phenol-chloroform procedure (17). DNAs were digested with *Bgl* II, electrophoresed in 0.8% agarose gels, transferred onto nylon membranes (GeneScreen, NEN), and hybridized with [α-^{32}P]dCTP-labeled DNA probes. For Northern blot analysis, 20 μg of total RNA per lane was size-separated in 1% agarose/2.2 M formaldehyde gels, blotted onto nylon membranes (Schleicher & Schuell), hybridized overnight with radiolabeled DNA probes, and washed at high stringency. Autoradiograms exposed within the linear range of the film (Kodak) were quantified by scanning densitometry to assess gene amplification and levels of RNA expression. Reverse transcriptase–PCR was performed as previously described (18). The following conditions were used for amplification of *AKT2*: 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min for 35 cycles; after the final cycle, the reaction was held at 72°C for 10 min. As a control, a portion of the glyceraldehyde-3-phosphate dehydrogenase gene was also amplified using the same PCR conditions described above. The resulting PCR products were electrophoresed on a 1.5% agarose gel. The primers used for a portion of the open reading frame of *AKT2*

Abbreviations: AS, antisense; S, sense.

§To whom reprint requests should be addressed.

were 5′-ATGAATGAGGTGTCTGTCAT-3′ and 5′-GCTGAGGAAGAACCTGTG-3′.

Plasmid Construction and DNA Transfection. A 1.2-kb *Eco*RI fragment of a human *AKT2* cDNA clone (λHTakt-6) was ligated into a LXSN retroviral vector (19) in both the sense (S) and antisense (AS) orientations. The fragment was generated by deleting 70 aa from the C-terminus of the open reading frame of *AKT2*, followed by ligation to the *Eco*RI site of the LXSN vector, to obtain LXSN-AKT2-AS and LXSN-AKT2-S constructs. The promoter within the vector, from the 5′ long terminal repeat of the Moloney murine leukemia virus, is capable of constitutively directing the synthesis of RNA from the inserted DNA. The LXSN vector has a neomycin resistance gene suitable for selection of the transfectants. Lipofectin reagent (GIBCO/BRL) was used to transfect PANC1 cells with 10 μg of LXSN-AKT2-AS, LXSN-AKT2-S, or LXSN vector DNA according to the manufacturer's instructions. Forty-eight hours after transfection, G418 was added to the medium at a concentration of 500 μg/ml. Individual colonies were isolated and grown in culture for further analysis. Five clonal sense and five clonal antisense cell lines were established, and these same clones were characterized for *in vitro* growth, morphology, and tumorigenicity.

Fluorescence *in Situ* Hybridization. Metaphase spreads from the ductal pancreatic carcinoma cell line PANC1 transfected with the LXSN-AKT2-AS construct were prepared according to standard procedures. Fluorescence *in situ* hybridization was carried out basically as described by Fan *et al.* (20). The metaphase spreads were co-hybridized to nonisotopically labeled *AKT2* cDNA fragment (1.4 kb) and LXSN vector probes. The *AKT2* probe was labeled with biotin-16-dUTP (Boehringer Mannheim), and hybridization was detected with fluorescein-conjugated avidin. Hybridization of digoxigenin-11-dUTP-labeled LXSN probe was detected with anti-digoxigenin-rhodamine (Boehringer Mannheim). Chromosomes were stained with 4′,6-diamidino-2-phenylindole dihydrochloride in antifade solution (Oncor) and examined by fluorescence microscopy. Metaphase spreads were observed by use of a Zeiss Axiophot microscope. Images were captured by a cooled charge-coupled device camera (Photometrics, Tucson, AZ) connected to a computer work station. Digitized images of fluorescein signals and counterstained chromosomes were merged as described (21).

Western Blot Analysis. Protein extracts were prepared by lysing cells in Triton extraction buffer (10 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM EDTA/10% glycerol/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/aprotinin at 1 μg/ml/1 mM sodium orthovanadate). Equal amounts of protein (50 μg) were boiled in Laemmli sample buffer, separated on SDS/12% polyacrylamide gel, and transferred to Immobilon-p (Millipore) in Bjerrum and Schafer–Nielsen buffer (48 mM Tris, pH 9.2/39 mM glycine) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Filters were blocked overnight in 10 mM Tris, pH 8.0/0.9% NaCl/0.05% Tween 20 plus 0.02% NaN_3 and 1.0% bovine serum albumin (Sigma, fraction V) at 4°C. AKT2 protein was detected with a polyclonal antibody raised against the pleckstrin homology domain of AKT2 by use of the enhanced chemiluminescence Western blotting analysis system (Amersham).

Tumorigenicity in Nude Mice. Tumorigenicity of the pancreatic carcinoma cell lines was examined by s.c. inoculation of 1.5×10^5 or 1×10^6 cells in 4-week-old female *nude/nude* mice. Each of five different clonal cell lines containing the antisense *AKT2* construct and five clonal lines with the sense construct was injected into three animals at 1.5×10^5 cells/mouse and another three animals at 1×10^6 cells/mouse (total, 60 mice). Tumor formation was assessed over a 3-month period following s.c. inoculation. Tumor measurements were made with linear calipers in two orthogonal directions by the same observer.

Intratracheal Inoculation and Xenotransplantation of Carcinoma Cell Lines. To examine further the effect of antisense *AKT2* on tumorigenicity, we used an intratracheal inoculation and xenotransplantation assay (22) to examine tumor growth and invasiveness of three human pancreatic carcinoma cell lines, including two (ASPC1 and PANC1) with amplified *AKT2* and a third (COLO 357) that does not overexpress this gene. Transfection of the three cell lines with LXSN-AKT2-AS DNA was performed as described above. Mass cell populations were selected, without cloning, in medium containing G418 for 4 weeks prior to xenotransplantation.

The tracheal transplants were prepared as previously described (22). The cell lines were harvested enzymatically when semiconfluent, and 5×10^5 cells of each parental and transfected cell line were inoculated into deepithelialized Fischer 344 rat tracheas. The tracheas were sealed at each end with clips and then transplanted into the dorsal s.c. tissue of skid mice (each mouse was xenotransplanted with two tracheas; two mice/cell line). The tracheal transplants were surgically removed at 4 and 6 weeks after xenotransplantation, fixed in neutral buffered formalin, and embedded in paraffin. Sections were obtained for histologic analysis. Invasion of the wall was classified according to the level of penetration of the neoplastic cells (22).

RESULTS

Amplification and Overexpression of *AKT2* in Pancreatic Carcinomas. Southern blot analysis was performed on 18 pancreatic cancer cell lines and 10 primary ductal tumors. Two cell lines, ASPC1 and PANC1, showed 30-fold and 50-fold amplification of *AKT2*, respectively, and one tumor specimen displayed 6-fold amplification of this gene (Fig. 1). This amplification does not involve the DNA repair gene *ERCC1*, which is located at 19q13.2–q13.3, near the *AKT2* locus (19q13.1–q13.2), indicating that the increased *AKT2* copy number is not due to polysomy 19. Northern blot analysis revealed highly elevated *AKT2* transcript levels in ASPC1 and PANC1 compared with that observed in other pancreatic tumor cell lines (Fig. 1). Western blot analysis demonstrated abundant AKT2 in PANC1 and ASPC1 cells relative to carcinoma cell lines in which *AKT2* was not amplified (Fig. 2).

Growth Inhibition of PANC1 Cells by Antisense *AKT2* RNA. To study the tumorigenic effect of *AKT2* overexpression, PANC1 cells were transfected with LXSN-AKT2-AS, LXSN-AKT2-S, and LXSN. Five clonal cell lines were obtained from each transfection. Integration of the antisense construct within the genome of cells transfected with LXSN-AKT2-AS was confirmed by fluorescence *in situ* hybridization. In the mass cell population, one to three integrations at variable locations (frequently near telomeres or centromeres) were observed in each of the metaphase spreads examined (Fig. 3). In the LXSN-AKT2-AS clones, the specific locations of the integration construct differed from cell line to cell line. In addition to *AKT2* signals at integration sites, multiple *AKT2*-specific hybridization signals were observed on a homogeneously staining region within a derivative chromosome 19, usually present in duplicate (Fig. 3). Southern blot analysis, using an LXSN retroviral probe and three different restriction enzymes to detect junction fragments, confirmed the independence of the five different antisense clones.

Each of the clonal cell lines transfected with the antisense construct had a similar morphology *in vitro* that differed markedly from that of parental PANC1 cells, the clones transfected with *AKT2* in the sense orientation, and clones transfected with LXSN. Cells transfected with LXSN-AKT2-AS were larger and flatter than parental cells or cells transfected with other constructs. At confluency, the parental PANC1 cells and LXSN-AKT2-S-transfected cells tended to "pile up" and, thus, reached higher densities than cells trans-

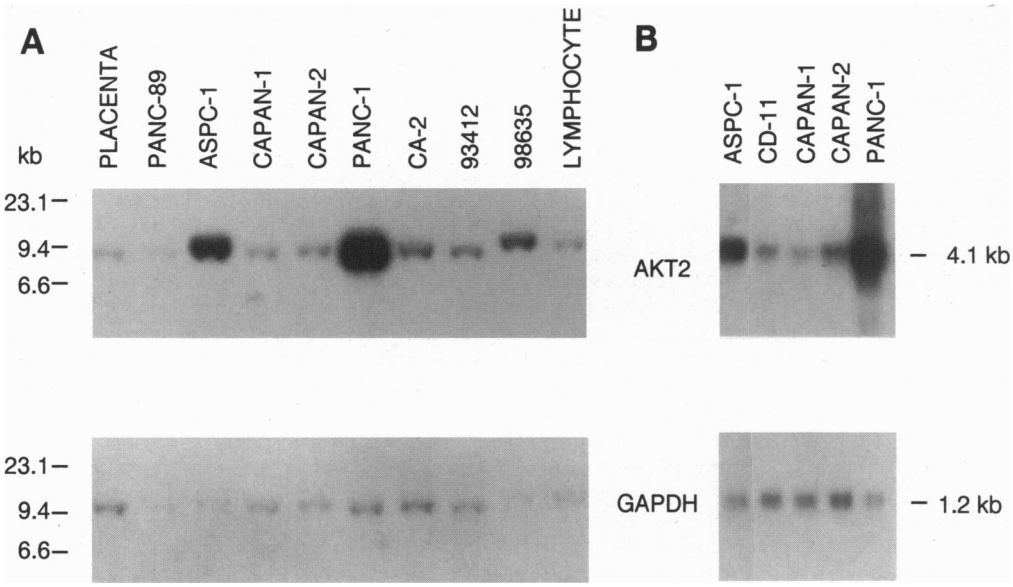

FIG. 1. Southern and Northern blot analyses of pancreatic carcinoma cell lines and primary tumors. (*A*) Southern blot analysis of *Bgl* II-digested DNA from five tumor cell lines and three primary tumors. (*Upper*) Hybridization to 2.0-kb genomic *AKT2* probe (1). Two cell lines and one primary tumor exhibit gene amplification (lanes 3, 6, and 9). (*Lower*) Rehybridization to a probe for *ERCC1*, which is also located on chromosome 19q. (*B*) Northern blot analysis. (*Upper*) Hybridization of a 1.4-kb *AKT2* cDNA probe (1) to total RNAs from five cell lines. Two cell lines displaying overexpression of *AKT2* (4.1-kb transcript, lanes 1 and 5) are identical to those having amplification. (*Lower*) Rehybridization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

fected with the antisense construct. However, there was no apparent difference in the population doubling times among the various cell lines (35–47 h in parental PANC1 and LXSN-AKT2-S-transfected cells, as compared with 33.5–45 h in the antisense clones).

Antisense RNA Blocks the Expression of AKT2 Protein. Antisense and sense *AKT2* PANC1 transfectants, parental PANC1 cells, and PANC1 cells transfected with LXSN vector alone were lysed for subsequent studies of AKT2 protein levels. Western blot analysis revealed that all five antisense clones showed a marked reduction in the amount of *AKT2* gene product compared with controls (Fig. 4). AKT2 protein was not detected in four antisense clones, whereas cell line LXSN-AKT2-AS-3 showed ≈90% reduction of AKT2 expression compared with control cells. However, reverse transcriptase–PCR analyses revealed that *AKT2* RNA appeared to be uniformly expressed in parental PANC1 cells and PANC1 cells transfected with either LXSN-AKT2-S, LXSN-AKT2-AS, or LXSN alone (data not shown).

Antisense *AKT2* RNA Inhibits Tumorigenicity of PANC1 in Nude Mice. Inoculation of five different clonal cell lines (three mice per clone) with sense construct at 1.5×10^5 cells/mouse produced poorly differentiated pancreatic tumors within 28 days in 2 of 15 nude mice. None of the 15 mice inoculated with the five antisense clones at 1.5×10^5 cells/mouse developed tumors during a 3-month observation period. When the inoculum was increased to 1×10^6 cells, tumors formed within 25 days in all 15 mice injected with the LXSN-AKT2-S clones but in only 2 of 15 mice inoculated with the LXSN-AKT2-AS clones. The latter two tumors were first observed 75 days after inoculation and were induced by the LXSN-AKT2-AS-3 cells, which exhibited ≈90% reduction of AKT2 protein (Fig. 4).

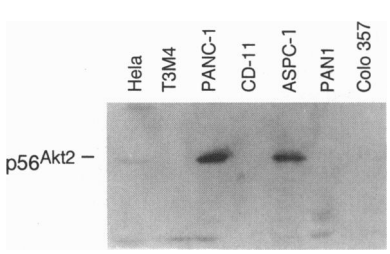

FIG. 2. Western blot analysis of cell lysates reacted with AKT2 polyclonal antibody. Note high levels of AKT2 in PANC1 and ASPC1 compared with other pancreatic carcinoma cell lines and HeLa cells.

Antisense *AKT2* RNA Inhibits Growth and Invasiveness of ASPC1 and PANC1 Cells, but Not COLO 357 Cells, Xenotransplanted in Tracheal Grafts. To demonstrate that the effects of antisense *AKT2* on PANC1 cells described above were not simply an isolated anomaly, an additional poorly differentiated pancreatic carcinoma cell line exhibited *AKT2* amplification (ASPC1) was examined, along with a pancreatic cancer cell line in which *AKT2* is neither amplified nor overexpressed (COLO 357). In addition, for each transfected cell line we used a pool of cells derived from several hundred G418-resistant colonies to eliminate the possibility of a clone-specific effect. Tracheal xenotransplants harvested at both 4 and 6 weeks showed that ASPC1 cells transfected with LXSN-AKT2-AS remained confined to the tracheal lumen and did not grow through the pars membranacea of the tracheal wall. Conversely, control parental ASPC1 cells showed a marked infiltration of the pars membranacea and by 6 weeks had invaded the outer peritracheal space (Fig. 5). Overall, PANC1 cells grew more slowly than ASPC1. Although the antisense-treated PANC1 cells remained in the tracheal lumen without reaching the pars membranacea, the parental PANC1 cells showed a more massive intralumenal growth that invaded the inner boundary of the pars membranacea by 6 weeks (data not shown). The COLO 357 cells transplanted in tracheal xenografts remained in the tracheal lumen and reached only the inner confine of the pars membranacea by 6 weeks. No difference was observed between the growth pattern of control and antisense-treated COLO 357 cells (Fig. 5).

DISCUSSION

Pancreatic cancer is the fourth leading cause of neoplastic deaths in the United States (23). Little is known about the molecular genetics of ductal pancreatic adenocarcinomas, although a high prevalence of *KRAS2, TP53, RB1*, and *CDKN2*

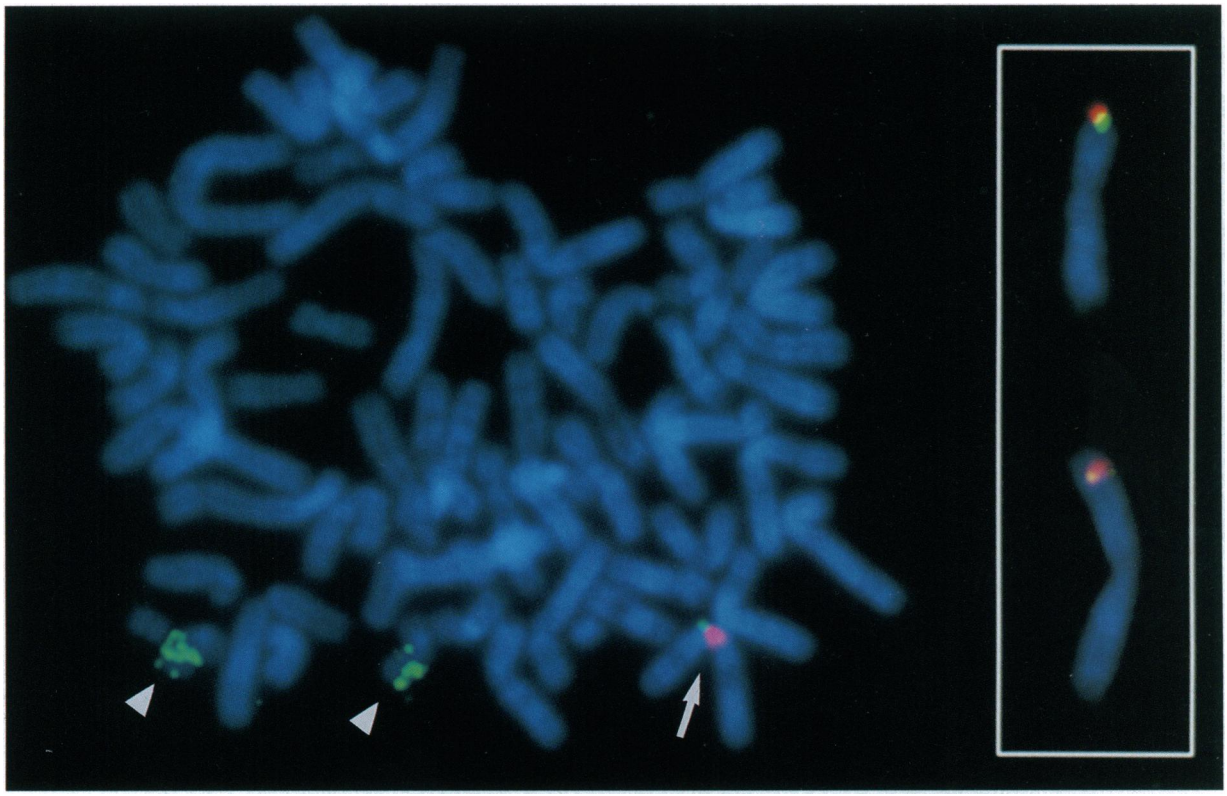

FIG. 3. Fluorescence *in situ* hybridization of *AKT2* cDNA and LXSN vector probes to a metaphase spread from a PANC1 cell transfected with LXSN-AKT2-AS. The *AKT2* probe was labeled with biotin and detected with fluorescein (green signals). The LXSN probe was labeled with digoxigenin and detected with rhodamine (red). Multiple *AKT2* signals (arrowheads) can be observed on homogeneously staining regions within two copies of a derivative chromosome 19, indicating that the amplified *AKT2* resides within these domains. Adjacent green and red signals or overlapping yellow areas indicate chromosomal integration site (arrow) of LXSN-AKT2-AS DNA. (*Inset*) Integration sites in two chromosomes from another metaphase spread.

(*p16*) mutations in these tumors has been described (24–27). Amplification of various oncogenes has been observed in many tumor types, but has rarely been reported in ductal pancreatic adenocarcinomas. In this study, amplification and/or overexpression of the putative oncogene *AKT2* were found in ≈10% of pancreatic cancer cell lines and primary tumor specimens.

We recently observed that overexpression of human *AKT2* can lead to transformation of NIH 3T3 cells (J.Q.C. and J.R.T., unpublished data). Gene amplification represents an important mechanism for increased expression of genes involved in tumorigenesis, and in some types of cancer amplification of specific oncogenes has been correlated with advanced disease (28–30). We previously demonstrated that *AKT2* is amplified and overexpressed in ≈15% of human ovarian carcinomas (1). A further study of 132 ovarian cancer specimens has revealed that *AKT2* alterations may be associated with a poor prognosis and are more frequent in undifferentiated ovarian carcinomas (8). PANC1 and ASPC1 were established from poorly differentiated tumors, and the primary pancreatic tumor specimen with amplified *AKT2* also was poorly differentiated. A study of a larger series of pancreatic cancer patients will be needed to determine whether *AKT2* alterations are associated with an advanced disease status in this malignancy.

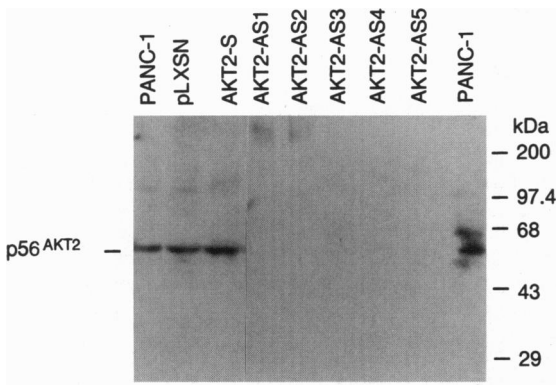

FIG. 4. Immunoblot of cell lysates reacted with AKT2 polyclonal antibody. Lanes 1 and 9, lysates of control PANC1 parental cells; lanes 2 and 3, lysates of PANC1 cells transfected with LXSN vector alone and with sense *AKT2* constructs, respectively; lanes 4–8, lysates of cells transfected with antisense *AKT2* constructs. Clone AKT2-AS-3 showed ≈90% reduction in the amount of AKT2 protein (detectable on original autoradiogram); no AKT2 protein was detected in the remaining four clones.

In this report, we demonstrate that antisense *AKT2* RNA can greatly inhibit the expression of AKT2 protein and suppress the tumorigenic phenotype of PANC1 cells inoculated s.c. in nude mice. Interestingly, the levels of AKT2 protein are associated with the rates of tumor growth in nude mice. Four PANC1 antisense clones (LXSN-AKT2-AS-1, -2, -4, and -5), without detectable AKT2 protein (Fig. 4), exhibited no tumor growth in nude mice. The remaining clone (LXSN-AKT2-AS-3), which displayed ≈90% reduction in the amount of AKT2 protein, formed tumors in two of six mice; both of these tumors occurred with the higher inoculum and required a longer latency than control cells transfected with the sense construct.

Intratracheal inoculation and xenotransplantation of human tumor cell lines is an efficient alternative to the s.c. inoculation assay for tumorigenicity and is also a potentially valuable approach for the study of tumor invasiveness and other aspects of neoplastic progression (22, 31). This assay was used to assess the effect of the antisense *AKT2* construct on tumor cell growth and invasiveness in mass populations of PANC1 and ASPC1 cells, both of which have amplified *AKT2*, and COLO

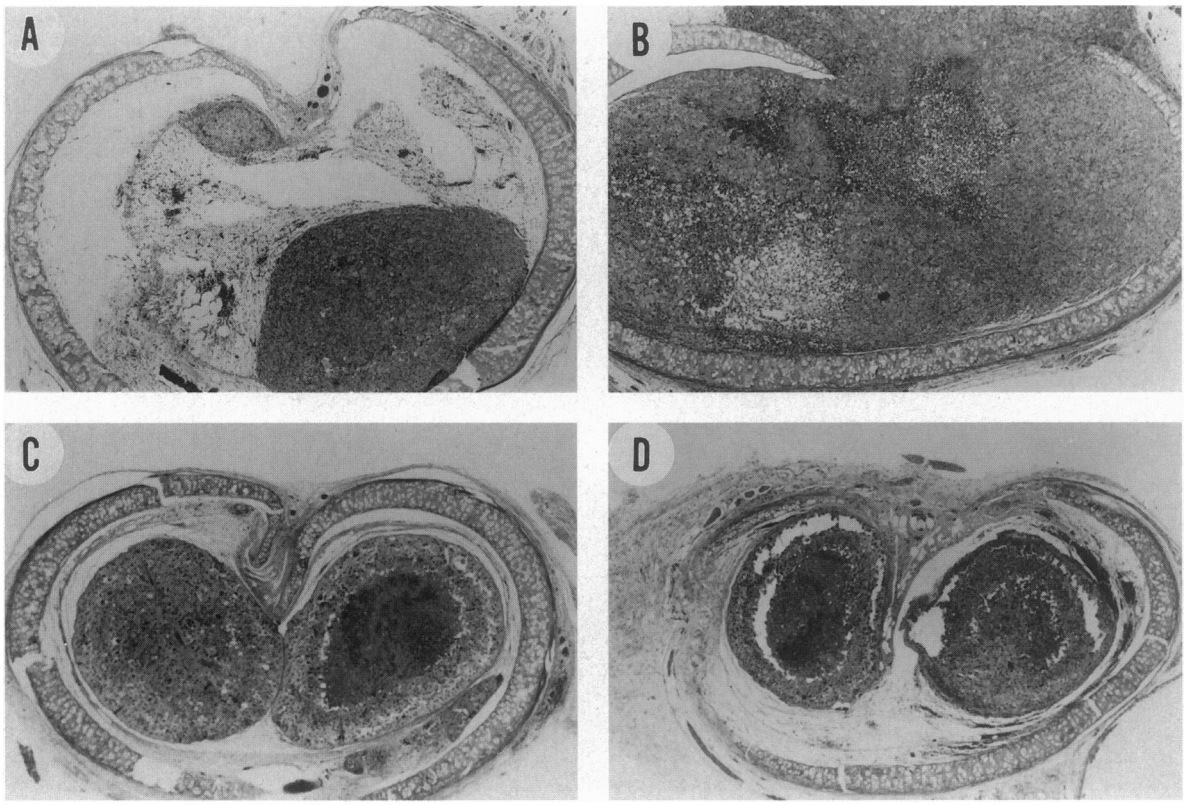

FIG. 5. Human pancreatic carcinoma cell lines xenotransplanted into tracheal grafts. At 6 weeks: (*A*) ASPC1 cells transfected with antisense *AKT2* remained localized within the trachea and did not invade the wall. (*B*) ASPC1 parental cells grew massively and invaded the pars membranacea and tissue outside the tracheal graft. (*C*) COLO 357 cells treated with antisense *AKT2* occupy the tracheal graft and show no difference in growth pattern from (*D*), parental COLO 357 cells in a similar tracheal transplant. (Hematoxylin/eosin; ×40.)

357 cells, which do not overexpress this gene. Antisense *AKT2* inhibited the tumorigenic growth of ASPC1 and PANC1. In contrast, the COLO 357 cells appeared to be refractory to the expression of antisense *AKT2* RNA. These data support the contention that the functional effect of antisense *AKT2* is restricted to cells that overexpress, and are therefore presumably dependent on, AKT2 protein for some of their tumorigenic properties. It is now known that members of the Akt family can be activated by various growth factors through phosphatidylinositol-3-OH kinase (5–7). Thus, it is possible that the overexpression of *AKT2* could upregulate the mediation of growth signals that may contribute to cell proliferation.

In addition to amplification of *AKT2*, both PANC1 and ASPC1 cells possess alterations of *TP53, KRAS2*, and *CDKN2* (refs. 24, 26, 27, 32, and B.R., unpublished data) as well as multiple chromosome abnormalities (Z. Liu and J.R.T., unpublished data). The fact that antisense RNA targeting *AKT2* alone can greatly inhibit proliferation of these tumor cells suggests that overexpressed *AKT2* plays an important role in the malignant phenotype of these cells. Similarly, *ERBB2* antisense oligonucleotides have been shown to inhibit the *in vitro* growth of breast carcinoma cell lines having *ERBB2* amplification but had no specific effect on cell lines that lacked amplification of this oncogene (16). These experimental data suggest that certain oncogenes and their products can be potential therapeutic targets in tumors exhibiting amplification and overexpression of such genes.

We thank Dr. Andres Klein-Szanto for preparing deepithelialized rat tracheas and for histologic assessment of human tumor cells xenotransplanted in tracheal grafts, Dr. Ze Min Liu for karyotypic analysis of the parental PANC1 cells, and Dr. Jerome Freed and Sharon Howard for assessment of *in vitro* growth rates of PANC1 and clonal derivatives. This research was supported by American Cancer Society Grant EDT-6, National Cancer Institute Grant CA-06927, and by an appropriation from the Commonwealth of Pennsylvania. J.Q.C. is a Special Fellow of the Leukemia Society of America. D.A.A. is supported by National Institutes of Health Postdoctoral Training Grant CA-09035 to Fox Chase Cancer Center.

1. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N. & Testa, J. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9267–9271.
2. Bellacosa, A., Testa, J. R., Staal, S. P. & Tsichlis, P. N. (1991) *Science* **254,** 274–277.
3. Mayer, B. J., Ren, R., Clark, K. L. & Baltimore, D. (1993) *Cell* **73,** 629–630.
4. Musacchio, A., Gibson, T., Rice, P., Thompson, J. & Saraste, M. (1993) *Trends Biochem. Sci.* **18,** 343–348.
5. Franke, T. F., Yang, S.-I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. & Tsichlis, P. N. (1995) *Cell* **81,** 727–736.
6. Burgering, B. M. T. & Coffer, P. J. (1995) *Nature (London)* **376,** 599–602.
7. Downward, J. (1995) *Nature (London)* **376,** 553–554.
8. Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Panici, P. B., Mancuso, S., Neri, G. & Testa, J. R. (1995) *Int. J. Cancer* **64,** 280–285.
9. Kasid, U., Pfeifer, A., Brennan, T., Beckett, M., Weichselbaum, R. R., Dritschilo, A. & Mark, G. E. (1989) *Science* **243,** 1354–1356.
10. Szczylik, C., Skorski, T., Nicolaides, N. C., Manzella, L., Malaguarnera, L., Venturelli, D., Gewirtz, A. M. & Calabretta, B. (1991) *Science* **253,** 562–565.
11. Stein, C. A. & Cheng, Y.-C. (1993) *Science* **261,** 1004–1011.
12. Gray, G. D., Hernandez, O. M., Hebel, D., Root, M., Pow-Sang, J. M. & Wickstrom, E. (1993) *Cancer Res.* **53,** 577–580.
13. Schmidt, M. L., Salwen, H. R., Manohar, C. F., Ikegaki, N. & Cohn, S. L. (1994) *Cell Growth Differ.* **5,** 171–178.
14. Francastel, C., Groisman, R., Pfarr, C. M. & Robert-Lezenes, J. (1994) *Oncogene* **9,** 1957–1964.

15. Georges, R. N., Mukhopadhyay, T., Zhang, Y., Yen, N. & Roth, J. A. (1993) *Cancer Res. Cell* **53,** 1743–1746.
16. Colomer, R., Lupu, R., Bacus, S. S. & Gelmann, E. P. (1994) *Br. J. Cancer* **70,** 819–825.
17. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
18. Cheng, J. Q., Jhanwar, S. C., Klein, W. M., Bell, D. W., Lee, W.-C., Altomare, D. A., Nobori, T., Olopade, O. I., Buckler, A. J. & Testa, J. R. (1994) *Cancer Res.* **54,** 5547–5551.
19. Miller, A. D. & Rosman, G. J. (1989) *BioTechniques* **7,** 980–990.
20. Fan, Y.-S., Davis, L. M. & Shows, T. B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6223–6227.
21. Testa, J. R., Taguchi, T., Knudson, A. G. & Hino, O. (1992) *Cytogenet. Cell Genet.* **60,** 247–249.
22. Momiki, S., Baba, M., Caamano, J., Iizasa, T., Nakajima, M., Yamaguchi, Y. & Klein-Szanto, A. (1991) *Invasion Metastasis* **11,** 66–75.
23. Boring, C. C., Squires, T. S. & Tong, T. (1993) *Ca Cancer J. Clin.* **43,** 7–26.
24. Ruggeri, B., Zhang, S.-Y., Caamano, J., DiRado, M., Flynn, S. D. & Klein-Szanto, A. J. P. (1992) *Oncogene* **7,** 1503–1511.
25. Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N. & Perucho, M. (1988) *Cell* **53,** 549–554.
26. Pellegata, N. S., Sessa, F., Renault, B., Bonato, M., Leone, B. E., Solcia, E. & Ranzani, G. N. (1994) *Cancer Res.* **54,** 1556–1560.
27. Caldas, C., Hahn, S. A., da Costa, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J. & Kern, S. E. (1994) *Nat. Genet.* **8,** 27–32.
28. Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E. & Bishop, J. M. (1984) *Science* **224,** 1121–1124.
29. Yokota, J., Tsunetsugu-Yokota, Y., Battifora, H., Le Fevre, C. & Cline, M. J. (1986) *Science* **231,** 261–265.
30. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. & McGuire, W. L. (1987) *Science* **235,** 177–182.
31. Baba, M., Klein-Szanto, A. J. P., Trono, D., Obara, T., Yoakum, G. H., Masui, T. & Harris, C. C. (1987) *Cancer Res.* **47,** 573–578.
32. Berrozpe, G., Schaeffer, J., Peinado, M. A., Real, F. X. & Perucho, M. (1994) *Int. J. Cancer* **58,** 185–191.