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AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas

(Src homology 2-like domain/gene amplification/chromosome alterations)

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ABSTRACT **We isolated cDNA clones containing the entire coding region of the putative oncogene *AKT2*. Sequence analysis and *in vitro* translation demonstrated that *AKT2* encodes a 56-kDa protein with homology to serine/threonine kinases; moreover, this protein contains a Src homology 2-like domain. *AKT2* was shown to be amplified and overexpressed in 2 of 8 ovarian carcinoma cell lines and 2 of 15 primary ovarian tumors. *AKT2* was mapped to chromosome region 19q13.1–q13.2 by fluorescence *in situ* hybridization. In the two ovarian carcinoma cell lines exhibiting amplification of *AKT2*, the amplified sequences were localized within homogeneously staining regions. We conclude that AKT2 belongs to a distinct subfamily of protein-serine/threonine kinases containing Src homology 2-like domains and that alterations of *AKT2* may contribute to the pathogenesis of ovarian carcinomas.**

akt, the protooncogene transduced by the acute transforming retrovirus AKT8 (1, 2), encodes a protein-serine/threonine kinase containing a Src homology 2-like (SH2-like) domain (3). Two putative human cellular homologs, *AKT1* and *AKT2*, were cloned by screening a human genomic DNA library with a v-*akt* probe under conditions of reduced stringency (4). Recently, we obtained *AKT1* clones from a normal human thymus cDNA library by using an *AKT1* genomic probe. Sequence analysis of portions of *AKT1* cDNA clones revealed that *AKT1* is the true human homolog of v-*akt* and is identical to the recently cloned *RAC* gene, which has been shown to encode a kinase related to members of the protein kinase C (PKC) family and the cyclic adenosine monophosphate-dependent protein kinase (cAMP-PK) (5). *AKT1* has been mapped to human chromosome band 14q32 (6), proximal to the immunoglobulin heavy-chain locus, and has been shown to be amplified in a gastric adenocarcinoma (4).

In this communication, we report the cDNA cloning, sequence analysis,† and chromosomal mapping of *AKT2* and demonstrate that this putative oncogene encodes a protein belonging to a subfamily of serine/threonine kinases containing SH2-like domains. Moreover, we show that *AKT2* is amplified and overexpressed in some human ovarian carcinoma cell lines and primary tumors, suggesting that it contributes to the development of common epithelial tumors of the ovary.

MATERIALS AND METHODS

Ovarian Carcinoma Cell Lines and Primary Tumors. The ovarian carcinoma cell lines examined in this study have been described (7–9). Primary tumor specimens were obtained from patients who underwent surgery at Fox Chase Cancer Center.

cDNA Cloning and Sequencing. A human cDNA library in λgt11 derived from normal thymus cells (Clontech) was screened with a 2.0-kilobase (kb) *Sma* I–*Bam*HI *AKT2* genomic probe (4). The hybridization was carried out under conditions of high stringency; the final wash was at 65°C for 30 min in 0.1× SSC/0.1% SDS (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0). Positive clones were plaque-purified, and the *Eco*RI inserts were subcloned into pBluescript SK(−) (Stratagene) for further analysis. A 1.8-kb DNA fragment from the largest clone, λHTakt-6, was subjected to nested deletions in both directions, using the exonuclease III–mung bean nuclease method (Stratagene). Plasmid clones with overlapping deletions were sequenced. The sequencing reactions were performed on alkali-denatured double-stranded DNA by using the dideoxynucleotide chain-termination method (10) and the Sequenase version 2.0 system (United States Biochemical). The sequence of the 1.8-kb fragment was determined for both strands. Sequence analysis was carried out with the University of Wisconsin Genetics Computer Group software package (11).

***In Vitro* Transcription and Translation.** The *Eco*RI insert from λHTakt-6 was subcloned into pBluescript SK(−); the linearized plasmid was used as a template for *in vitro* transcription by T3 RNA polymerase. The capped RNA (2 μg) was translated *in vitro* by using rabbit reticulocyte lysate (Stratagene) for 1 hr at 30°C in the presence of 20 μCi (740 kBq) of [^{35}S]methionine. The samples were analyzed by SDS/15% PAGE followed by autoradiography for 12 hr at room temperature.

Southern and Northern Analysis. DNA from normal tissues, tumor specimens, and cell lines was isolated by standard techniques (12). Digested DNA, after careful spectrophotometric measurement of DNA concentration, was fractionated by 1.0% agarose gel electrophoresis, and transferred to nylon membranes (GeneScreen*Plus*; NEN/DuPont) (12). The membranes were hybridized overnight with ^{32}P-labeled probes and washed at high stringency. Chromosome-mapping-panel blots containing human, hamster, and human–hamster somatic cell hybrid DNAs (Bios, New Haven, CT) were hybridized according to the manufacturer's instructions.

Total cellular RNA was obtained by a one-step guanidinium isothiocyanate/phenol/chloroform extraction procedure (13). For Northern analysis, 15 μg of total RNA per lane was

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Abbreviations: SH2-like, Src homology 2-like; PKC, protein kinase C; cAMP-PK, cyclic adenosine monophosphate-dependent protein kinase; FISH, fluorescence *in situ* hybridization.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M95936).

electrophoresed in a 1% agarose/2.2 M formaldehyde gel, blotted onto Magna NT membrane filters (Micron Separations, Westborough, MA), and hybridized overnight with ^{32}P-labeled probes.

Autoradiograms exposed within the linear range of the film were quantified by scanning densitometry (UltraScan XL laser densitometer; Pharmacia LKB) to assess gene amplification and levels of RNA expression.

Fluorescence *in Situ* Hybridization (FISH). Metaphase spreads were prepared from normal lymphocyte cultures and from ovarian carcinoma cell lines OVCAR-3 (passage 51) and OVCAR-8 (passage 8), following cell cycle synchronization with 5-bromodeoxyuridine (14). FISH was carried out basically according to Fan *et al.* (14), with a nonisotopically labeled 2.0-kb genomic *AKT2* fragment as probe. The probe was prepared by nick-translation (BRL) using biotin-11-dUTP (Enzo Diagnostics, New York). Hybridization was detected with fluorescein isothiocyanate-conjugated avidin. Chromosomes were counterstained with propidium iodide and 4′,6-diamidino-2-phenylindole dihydrochloride and observed under fluorescence microscopy. Metaphase spreads were photographed on Kodak Ektachrome 400 film.

RESULTS

Cloning and Sequence Analysis of *AKT2* cDNA. Three clones, λHTakt-3, -6, and -8, were isolated by screening a normal human thymus cDNA library with an *AKT2* genomic probe (4). λHTakt-6 contains the entire open reading frame (1443 base pairs) and overlaps the λHTakt-3 and -8 clones. Sequence analysis revealed that *AKT2* encodes a 481-amino acid-long protein-serine/threonine kinase containing an SH2-like domain (Fig. 1) (3, 15, 16). The putative catalytic domain of the AKT2 kinase has the 11 conserved subdomains characteristic of protein kinases (17) and is closely related to human PKCα (68.1% similarity, 47.9% identity) (18), rat PKCδ (67.2% similarity, 44.0% identity) (19), rabbit PKCγ (65.3% similarity, 44.9% identity) (20), and human cAMP-PKβ (64.8% similarity, 42.8% identity) (21). The predicted protein encoded by the *AKT2* cDNA contains the sequence motifs Gly-Xaa-Gly-Xaa-Xaa-Gly-$(Xaa)_{16}$-Lys (amino acid positions 159–181) and Asp-Phe-Gly (positions 293–295), matching the consensus sequence for ATP-binding sites. Also present are the sequences Gly-Thr-Pro-Glu-Tyr-Leu-Ala-Pro-Glu (positions 312–320) and Asp-Ile-Lys-Leu-Glu-Asn (positions 275–280), which match the consensus sequences that distinguish protein-serine/threonine kinases from protein-tyrosine kinases (5, 17).

The *AKT2* gene is highly homologous to v-*akt* and *AKT1/RAC*. The homology of *AKT2* to *AKT1/RAC* and the nonviral portion of v-*akt* is 77.6% and 77.5%, respectively, at the nucleotide level and 90.6% and 90.4%, respectively, at the amino acid level. In addition, their protein products are predicted to share the same overall structure. AKT2 contains an SH2-like domain that extends over a stretch of 77 amino acids (amino acids 2–78). The similarity of the SH2-like domain of AKT2 to those of v-Akt and AKT1/RAC is high (80.5% and 81.8% identity, respectively). Between the SH2-like domain and the catalytic domain, each of these proteins contains a region rich in glutamic and aspartic residues (13 out of 70 residues, 18.6%, in AKT2), the amino-terminal portion of which is predicted to form an amphipathic helix. The AKT2 catalytic domain contains a 70-amino acid carboxyl-terminal tail also found in v-Akt, AKT1/RAC, and members of the PKC family of kinases (3, 5, 17).

***In Vitro* Translation of *AKT2*.** To confirm the open reading frame, *AKT2* RNA was synthesized *in vitro* and translated in a cell-free system in the presence of [^{35}S]methionine. The sense transcript produced a 56-kDa protein (Fig. 2), in good agreement with the size predicted by sequence analysis (M_r 55,832). This verifies that the first ATG codon at the 5′ end of the *AKT2* open reading frame is the correct site of the initiator methionine residue.

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    AKT2    1 MNEVSVIKEGWLHKRGEYIKTWRPRYFLLKSDGSFIGYKE 40
AKT1/RAC      .SD.AIV.....................N..T......
   v-akt      ..D.AIV.....................N..T......

    AKT2   41 RPEAPDQTLPPLNNFSVAECQLMKTERPRPNTFVIRCLQW 80
AKT1/RAC      ..QDV..REA.....Q.............I......
   v-akt      ..QDV..RES.....Q.............I......

    AKT2   81 TTVIERTFHVDSPDEREEWMRAIQMVANSLKQRAPGEDPM 120
AKT1/RAC      ..........ET.E.....TT...T..DG..KQ--E.EE.
   v-akt      ..........ET.E.....AT...T..DG..RQ--E.ET.
                                          Catalytic
    AKT2  121 DYKCGSPSDSSTTEEMEVAVSKARAKVTMNDFDYLKLLGK 160
AKT1/RAC      .FRS.....N.GA.....SLA.PKHR....E.E.......
   v-akt      .FRS.....N.GA.....SLA.PKHR....E.E.......
              domain
    AKT2  161 GTFGKVILVREKATGRYYAMKILRKEVIIAKDEVAHTVTE 200
AKT1/RAC      ......................K....V........L..
   v-akt      ......................K....V........L..

    AKT2  201 SRVLQNTRHPFLTALKYAFQTHDRLCFVMEYANGGELFFH 240
AKT1/RAC      N.....S..........S.....................
   v-akt      N.....S..........S.....................

    AKT2  241 LSRERVFTEERARFYGAEIVSALEYLHS-RDVVYRDIKLE 279
AKT1/RAC      .......S.D.............D....EKN.....L...
   v-akt      .......S.D.............D....EKN.....L...

    AKT2  280 NLMLDKDGHIKITDFGLCKEGISDGATMKTFCGTPEYLAP 319
AKT1/RAC      ......................K.................
   v-akt      ......................K.................

    AKT2  320 EVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHERLF 359
AKT1/RAC      ....................................K..
   v-akt      ....................................K..

    AKT2  360 ELILMEEIRFPRTLSPEAKSLLAGLLKKDPKQRLGGGPSD 399
AKT1/RAC      ..............G.......S.............SE.
   v-akt      ..............G.......S.......T......SE.

    AKT2  400 AKEVMEHRFFLSINWQDVVQKKLLPPFKPQVTSEVDTRYF 439
AKT1/RAC      ...I.Q....AG.V..H.YE...S.........T.....
   v-akt      ...I.Q....AN.V....YE...S.........T.....

    AKT2  440 DDEFTAQSITITPPDRYDSLGLLELDQRTHFPQFSYSASI 479
AKT1/RAC      .E.....M.......QD..MECVDSER.P.........S
   v-akt      .E.....M.......QD..MECVDSER.P.........G

    AKT2  480 RE 481
AKT1/RAC      TA
   v-akt      TA
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FIG. 1. Predicted protein sequence of *AKT2* cDNA and comparison with the amino acid sequences of AKT1/RAC and v-Akt. The SH2-like domain is boxed, and the catalytic region is identified by an arrow. Components of the ATP-binding sites are indicated by lines above and below symbols of individual amino acids. Sequences conserved in protein-serine/threonine kinases are underlined. Abbreviations for the amino acid residues are as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine. Dots indicate residues identical to the AKT2 sequence, and dashes indicate gaps introduced for alignment.

Expression of *AKT2* in Human Cells. Expression of *AKT2* in a variety of normal and neoplastic human cells was evaluated by Northern blot analysis. Total RNA isolated from spleen tissue, Epstein–Barr virus-immortalized B cells, and a total of 25 tumor specimens and tumor cell lines from patients with various types of cancer was examined. The *AKT2* message was expressed in all specimens and cell lines tested (data not shown). The size of the *AKT2* message is 4.1 kb. High levels of expression of *AKT2* mRNA were observed in two ovarian carcinoma cell lines (OVCAR-3 and OVCAR-8) (Fig. 3). These cell lines exhibited ≈30-fold and ≈45-fold increases in *AKT2* mRNA levels, respectively, compared with cultured diploid human ovarian surface epithelial (HOSE) cells in early passage.

Amplification of *AKT2* in Ovarian Carcinomas. Southern blot analysis revealed ≈10-fold and ≈15-fold amplification of

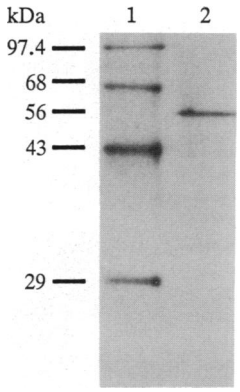

FIG. 2. *In vitro* translation of the AKT2 protein. Lane 1, ^{14}C-labeled protein molecular size standards; lane 2, ^{35}S-labeled 56-kDa protein synthesized by *in vitro* translation of *AKT2* RNA.

AKT2 in OVCAR-3 and OVCAR-8, respectively (Fig. 3). Interestingly, among the remaining six ovarian carcinoma cell lines examined, five exhibited a decrease in the gene dosage for *AKT2*. We also evaluated DNA isolated from primary common epithelial tumors of the ovary for changes in gene dosage. Two of the 15 primary ovarian carcinomas (UPN 46 and 53) showed *AKT2* gene amplification (3-fold and 5-fold, respectively) compared with normal human placental DNA (Fig. 3 and data not shown). This amplification does not involve the DNA repair gene *ERCC1* (Fig. 3), which is located at 19q13.2–q13.3 (23, 24), near the *AKT2* locus (see below), suggesting that the increased *AKT2* copy number is not due to polysomy 19. Subsequent Northern blot analysis revealed overexpression of *AKT2* in these two primary tumors (data not shown), but accurate quantification of the levels of expression was not possible due to partial degradation of the mRNA in these specimens. Hybridization of Southern blots with a c-*erbB*-2/*HER2* cDNA probe revealed no significant change in gene dosage for any of the ovarian carcinoma cell lines and primary tumors examined (data not shown).

Chromosomal Localization of *AKT2* in Normal Human Cells and Ovarian Carcinomas. We initially mapped *AKT2* to chromosome 19 by using a mapping-panel blot. Twenty-five hamster–human somatic cell hybrids were screened for the presence of human *AKT2* gene sequences. The presence or absence of chromosomes found within these hybrids showed high levels of discordance for all human chromosomes except chromosome 19. Chromosome 19 was discordant with the presence of *AKT2* sequences in only 1 of 25 hybrids.

To confirm these results and more precisely localize the *AKT2* gene, we hybridized a nonisotopically labeled *AKT2* genomic probe to normal human metaphase spreads *in situ*. A total of 36 metaphase and prometaphase spreads were examined by FISH. Fluorescent signals were detected on chromosome 19 in 30 of 36 cells. Overall, 73 of 125 signals (58%) were located on chromosome 19. Among all metaphase spreads with signals on chromosome 19, labeling on both homologs of this chromosome was observed in 23 of 30 cells (77%). All signals on chromosome 19 were located at sub-band 19q13.1 or at the interface between sub-bands 19q13.1 and 19q13.2 (Fig. 4 *A* and *B*).

Karyotypic analysis of OVCAR-3 revealed many numerical and structural abnormalities, including a marker chromosome containing a large homogeneously staining region (HSR). OVCAR-8 also displayed a complex karyotype, including two different chromosomes with smaller HSRs. FISH of an *AKT2* genomic probe to metaphase spreads from OVCAR-3 and OVCAR-8 showed multiple fluorescein signals on each of these HSRs, demonstrating that the amplified *AKT2* sequences reside within these cytogenetic alterations (Fig. 4 *C* and *D*).

DISCUSSION

In this paper we report the characterization of *AKT2*, a gene encoding a protein-serine/threonine kinase related to PKC and cAMP-PK. The AKT2 protein is closely related to the

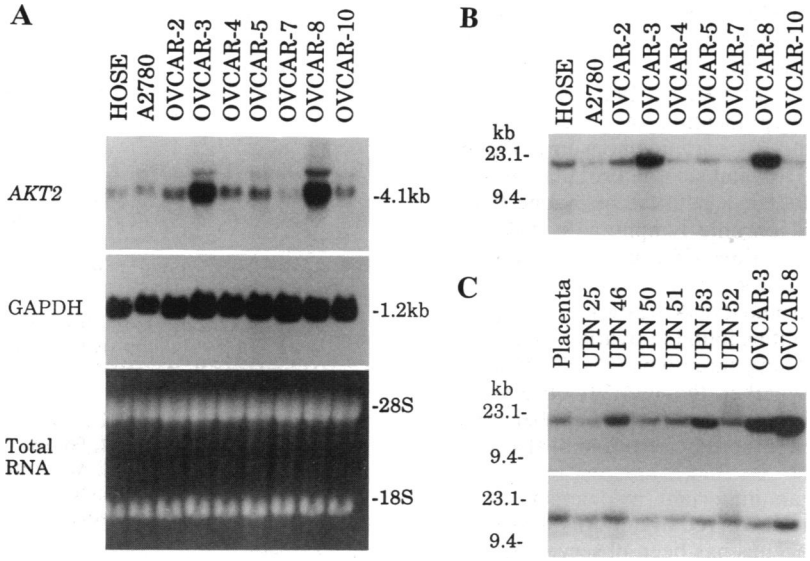

FIG. 3. Northern and Southern blot analysis of ovarian carcinoma cell lines and primary tumors. (*A*) Northern analysis of eight ovarian carcinoma cell lines. (*Top*) Hybridization of the blot with the *AKT2* cDNA probe. (*Middle*) Rehybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. (*Bottom*) Ethidium bromide-stained gel; positions of 28S and 18S rRNA are marked. *Middle* and *Bottom* demonstrate equivalent amounts of RNA in each lane. (*B* and *C*) Southern blot analysis of *Eco*RI-digested DNA from the same cell lines (*B*) and from primary ovarian carcinomas (*C*). *B* and *C Upper* show hybridization with a genomic *AKT2* probe. *C Lower* shows rehybridization with a probe for *ERCC1* (22). The 0.94-kb *ERCC1* cDNA probe detects four major bands (18, 3.6, 2.3, and 1.0 kb); the 18-kb band is closest in size to the *AKT2* band (21 kb) and is shown for comparison. *AKT2* amplification was evaluated in four separate experiments using three different restriction enzymes.

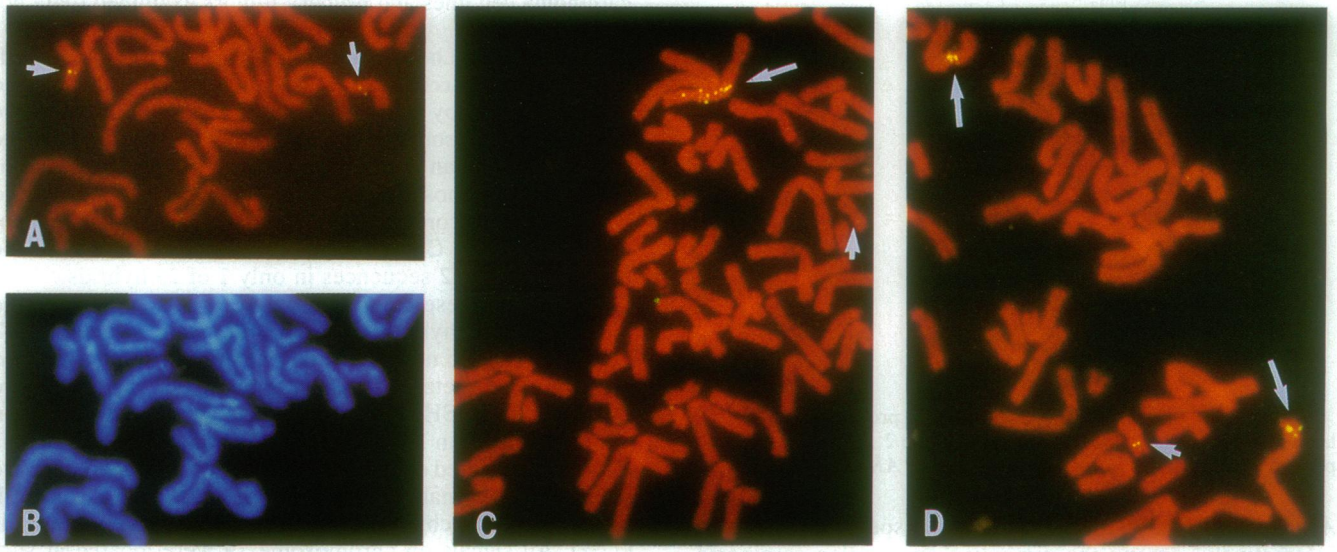

FIG. 4. FISH of *AKT2* to human metaphase chromosomes. (*A*) Localization of fluorescein-labeled probe on propidium iodide-stained normal lymphocyte metaphase spread. Fluorescent hybridization signals are indicated by arrows at 19q13.1–q13.2. (*B*) 4′,6-Diamidino-2-phenylindole fluorescence of the same spread, showing a Giemsa-like banding pattern useful to confirm the identity of individual chromosomes (14). (*C* and *D*) Localization of *AKT2* on propidium iodide-stained chromosomes from ovarian carcinoma cell lines OVCAR-3 (*C*) and OVCAR-8 (*D*). Fluorescent hybridization signals are indicated at 19q13.1–q13.2 (shorter arrows) and within homogeneously staining regions (longer arrows).

v-Akt and AKT1/RAC kinases, which also contain an SH2-like region amino-terminal to the kinase domain (3). The SH2-like regions of AKT2, AKT1/RAC, and v-Akt/c-Akt display similarities to the SH2 domains characteristic of cytoplasmic tyrosine kinases and other signaling proteins (3, 15). This suggests that the SH2-like domains may represent regions of protein–protein interactions that take place through the recognition of phospho amino acid residues. However, the similarity between SH2 and SH2-like domains is very distant, and the function of the SH2-like region may have diverged from that of SH2. The detection of this domain in both AKT1 and AKT2 places them into a distinct subfamily of protein-serine/threonine kinases. This subfamily may contain multiple genes, as suggested by Southern blot analysis of human genomic DNA hybridized to a c-*akt* SH2-like probe at low stringency (data not shown).

The chromosomal location of *AKT2* at 19q13.1–q13.2 is in close proximity to the map location of genes encoding transforming growth factor β1, carcinoembryonic antigen, the zinc finger protein ZFP36, and several proteins involved in DNA repair (22, 23). *BCL3*, a putative oncogene that is rearranged in some B-cell chronic lymphocytic leukemias displaying a 14;19 translocation (24), is also located at 19q13.1-q13.2 (23). In addition to the t(14;19) disrupting *BLC3*, the same 19q13 chromosomal region is the target of other cytogenetic abnormalities found in ovarian carcinoma, glioma, and non-small-cell lung carcinoma (25–27). It will be important to determine whether the t(14;19) observed in B-cell chronic lymphocytic leukemia or the other aberrations of the 19q13 region seen in other neoplasms affect the expression of *AKT2*.

Gene amplification is an important mechanism for increased expression of genes involved in tumorigenesis. Amplification of various oncogenes has been observed in many primary tumors and tumor cell lines (28). In some types of cancer, amplification of specific oncogenes has been correlated with advanced disease (29–31). The amplification and overexpression of *AKT2* in some ovarian carcinoma cell lines and primary tumors suggest that *AKT2*, in cooperation with other protooncogenes and tumor-suppressor genes (32–36), contributes to the pathogenesis of ovarian cancer. Further studies are needed to determine whether amplification of *AKT2* occurs in a significant proportion of other human neoplasms and whether such amplification represents a useful prognostic marker.

Note Added in Proof. Jones *et al.* (37) recently cloned a gene, rac protein kinase β, which encodes a protein highly related to PKC and cAMP-PK. A portion of the rac protein kinase β DNA sequence is identical to *AKT2*, but the regulatory region and the 3′ end of the open reading frame show considerable differences from those of *AKT2*.

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