PI3K/Akt Pathway Mutations in Retinoblastoma

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PURPOSE. Many malignancies are known to be associated with abnormal activation of the PI3K-AKT pathway. Recently, a somatic mutation in the AKT1 gene (E17K) was identified in a small proportion of human tumors. This mutation activated AKT1 by means of abnormal membrane recruitment and stimulated downstream signaling. This study was designed to analyze AKT1 mutations in retinoblastoma and gain insights into the role PI3K-AKT pathway plays in the development of this tumor.

METHODS. Twenty-four samples of retinoblastoma from children were analyzed for mutations in the AKT1, PTEN and K-RAS genes, using a chip-based matrix-assisted laser desorption-time-of-flight (MALDI-TOF) mass spectrometer. Mutations in the PIK3CA gene were analyzed in 16 retinoblastoma samples using direct sequencing.

RESULTS. These results show that the mutation E17K/AKT1 was not detected in the 24 samples of retinoblastoma analyzed. K-RAS mutations were identified in two samples. There were no mutations in any of the other genes analyzed by a mass array system. On direct sequencing of 16 samples for the PIK3CA gene, one sample showed gain of function mutation in exon 9. In another sample, a genetic polymorphism of unknown significance (rs17849079) was detected in exon 20.

CONCLUSIONS. Although the PI3K-AKT pathway is known to be dysregulated in retinoblastoma, the low frequency of oncogenic mutations in the AKT1, PIK3CA, and PTEN genes, suggests a different activating mechanism. (Invest Ophthalmol Vis Sci. 2009;50:5054–5056) DOI:10.1167/iovs.09-3617

Retinoblastoma, a tumor which arises from retinoblasts in the developing retina, is associated with inactivation of the retinoblastoma susceptibility gene, RB1, on chromosome band 13q14 in both heritable and non-heritable tumors.1,2

RB1 encodes for the retinoblastoma protein, Rb, which functions as a tumor suppressor by controlling the cell cycle through complex interactions of multiple kinases and their inhibitors, which together form the Rb pathway.3

The phosphoinositide 3-kinase (PI3K)/v-akt murine thymoma (Akt) viral oncogene pathway, is involved in regulating the signaling of multiple biological processes such as apoptosis, metabolism, cell proliferation, and cell growth.5 Its abnormal activation, frequently observed in various types of cancer, leads to aberrant cell cycle progression, altered adhesion and motility, inhibition of apoptosis, and induction of angiogenesis.6 Oncogenic events associated with abnormal activation of the PI3K/AKT pathway include mutations, allelic loss, or promoter methylation of the negative regulator phosphatase and tensin homolog (PTEN),7 as well as oncogenic mutations, chromosomal amplification or overexpression of the positive regulator phosphatidylinositol 3-kinase catalytic subunit (PIK3CA), which codes for the p110alpha catalytic subunit of PI3K,8,9 and various Akt kinases.10 PI3K/AKT pathway activation can also be induced by changes in other, related, pathways that are commonly altered in cancer, such as those involved in growth factor stimulation via the G-protein-coupled receptors or by direct interaction with the activated form of small GTPase Ras.11

Studies have shown that Ras regulates several pathways that contribute to cellular transformation, including Ras/Raf signaling pathway.12 Alternatively, Ras directly activates lipid kinases specifically, the conversion of phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol (3,4,5)-triphosphate (PIP3) by PI3K. Ras-induced activation of PI3K activates the PI3K/AKT pathway.13 In a study of retinoblastoma, Bautista et al.14 found 30% of the activating mutations in the gene encoding for K-RAS, one of the three principle members of the Ras family.

PTEN is a crucial tumor suppressor in the PI3K/AKT pathway because it catalyzes the precise opposite reaction to PI3K, thereby inhibiting downstream signaling. PTEN is a dual protein/lipid phosphatase and its main substrate, PIP3, is, as noted above, the product of PI3K. An increase in PIP3 recruits AKT to the membrane, where it is activated by other kinases also dependent on PIP3.5 Many primary and metastatic human cancers are characterized by a loss of PTEN activity due to mutations, deletions, or promoter methylation silencing at high frequency.

Activating mutations in the PI3KCA gene have been described in human tumors, and evidence over the past year suggests that they play a pivotal role in cancer.15 The potential oncogenicity of the PI3Ks is supported by findings of mutations causing gain of function and overexpression of wild-type proteins. PIK3CA has been implicated in many different tumors, including breast cancer,16 thyroid tumors,17 and head and neck (including oral) squamous cell carcinoma.18,19

Recently, a somatic mutation in the AKT1 gene was identified in a small proportion of breast, colorectal, and ovarian cancers20; specifically, a guanine-to-adenine transition at nucleotide 49 that resulted in an amino acid swap of lysine for glutamic acid at residue 17 (E17K) in the pleckstrin homology domain. This mutation activated AKTI by means of abnormal membrane recruitment and stimulated downstream signaling.

The aim of the study was to investigate the frequency of the AKTI mutations in retinoblastoma using DNA extracted from

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tumors in 24 patients. We also analyzed the tumors for other oncogenic mutations in the PI3K/AKT pathway, namely, PTEN, PIK3CA, and K-RAS genes.

**METHODS**

**Patients and Tissues**

The study was approved by the local and national review board. The archives of the Ophthalmic Pathology Laboratory of Hadassah-Hebrew University Medical Center were searched for all samples of retinoblastoma since 1997. DNA was then extracted and analyzed for mutations.

**DNA Extraction**

DNA was isolated from 24 archival formalin-fixed, paraffin-embedded samples of retinoblastoma diagnosed from 1997 to 2001, as previously described. In brief, hematoxylin-eosin stained 10 μm-section slides were reviewed by an ocular pathologist, and areas with >75% of tumor cells were identified. Microdissections were performed using a no. 11 surgical blade from five consecutive 10 μm unstained paraffin sections of each block. After deparaffinization, the microdissected sections were incubated overnight in 1% SDS and proteinase K 0.5 mg/mL. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and dissolved in 50 μL distilled water, as previously described.

**Mutation Analysis**

The AKT1 gene was analyzed for the E17K mutation with chip-based matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (Fig. 1). We also searched for the most frequent mutations in the PTEN, K-RAS, PIK3CA genes according to the COSMIC database, as follows: PTEN: exon 5 (R130L, R130G, R130X, R130Q) and 7 (R233X), using MALDI-TOF mass spectrometry; K-RAS: exon 2 (G12D, G12V, G12A, G13D), using MALDI-TOF mass spectrometry; PIK3CA: exons 9 and 20, as most PIK3CA mutations are confined to these exons, using direct sequencing.

**MALDI-TOF Mass Spectrometry**

For MALDI-TOF mass spectrometer (Sequenom; San Diego, CA) analysis of mutations in the AKTI, PTEN, and K-RAS genes, we designed specific primers flanking the mutation sites and extension primers that bind adjacent to the mutation site, using assay design software (Sequenom). The amplification and extension primers used in this study are available on request. After amplification of the region of interest, a primer extension reaction was carried out. The reaction included sequence-specific hybridization and sequence-dependent termination that generated different products for the mutated and wild type alleles, each with a unique mass value. Genotyping was performed by spotting the extension products onto silicon chips preloaded with proprietary matrix (SpectroChip; Sequenom) which were subsequently read by the MALDI-TOF mass spectrometer.

**PCR Amplification and Direct Sequencing**

To detect mutations in the PIK3CA gene, DNA was extracted using standard sodium dodecyl sulfate (SDS)/proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Each PCR amplification was performed in a 50 μL reaction volume containing 150 ng of sample DNA as a template. The reaction was performed using specific primers for PI3K: for exon 9—forward primer: GATTGGTCTTTTCTGTCTCTG, reverse primer: CCACCTATCAATTTAACCA; for exon 20 (two sets of primers)—forward primer (1): TGGGGTAAGGGAAATCAAAAG, reverse primer (1): CCTATGCAATCGATCTCCAGG, reverse primer (2): TGGCATACTGCAAAGACC, reverse primer (2): GGGAATTTTTGTGTTTG. The PCR parameters were as follows: denaturation at 95°C for 5 minutes; 35 cycles of 1 minute at 95°C; annealing at 56 to 60°C for 1 minute and 1 minute at 72°C with Taq polymerase. The PCR product was amplified on 2% agarose gel and visualized with ethidium bromide staining. Direct sequencing of the PCR products (exon 9, 487 bp fragment; exon 20, 525 bp and 469 bp fragments) was performed with reagents and an analyzer (Big Dye Terminator Cycle Sequencing and ABI PRISM 3700 DNA Analyzer; Applied Biosystems, Foster City, CA).

**RESULTS**

Our results show that the mutation E17K/AKT1 was not detected in the 24 samples of retinoblastoma analyzed. K-RAS mutations were identified in two samples out of 24. There were no mutations in any of the other genes analyzed by the Sequenom mass array system.
Sixteen archival retinoblastoma samples were analyzed for mutations in exons 9 and 20 of the PIK3CA gene with PCR and direct sequencing. One mutation was detected in A1634G exon 9. A genetic polymorphism of unknown significance in PIK3CA, rs17849079 (C3075T), was detected in exon 20.

**DISCUSSION**

The aim of the study was to determine the presence of mutations in the AKTI gene in patients with retinoblastoma. In addition, we searched for mutations in the K-RAS, and PTEN, PIK3CA genes, which are known to affect the function of the PI3K/AKT pathway.

The AKT family of protein kinases is one of the best characterized targets of PI3Ks. The PI3K/AKT signal transduction pathway regulates many growth and survival mechanisms, including transcription, cell cycle progression, metabolism, apoptosis, invasion and metastasis. The role of AKTI activation in human cancer has been extensively studied, but its activation by mutations was only recently discovered. Carpten et al. and Tibes et al. reported the novel point mutation (E17K) of the AKTI gene in human breast, colorectal, and ovarian cancers, and demonstrated that it induces leukemia in mice. To evaluate whether AKTI mutations play a role in retinoblastoma tumorigenesis, we screened for mutations in the PI3K/AKT pathway. The possible role of a E17K AKTI mutation in retinoblastoma was investigated previously by Chakraborty et al. at the cDNA and protein levels in a total of 200 genes. The mean mRNA expression levels of PIK3CA and AKT1 were significantly higher in retinoblastoma tumor samples compared to mean expression levels in normal retinas samples, suggesting that the PI3K/AKT pathway is dysregulated in retinoblastoma.

The low frequency of oncogenic mutations in the, AKTI, PIK3CA, PTEN, and K-RAS genes in the present study suggests that other mechanism of pathway-activation, such as copy number variations, might be involved in the tumorigenesis of retinoblastoma. The present study was performed on DNA extracted from paraffin-embedded tissue. Therefore, analysis of fresh tumor tissue might be needed to confirm the low frequency of AKTI mutation in retinoblastoma.

**References**