# Tumor Genetics; AKT Function and Oncogenic Activity

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Genomic imbalances and perturbations of signal transduction pathways involved in cell cycling and survival are considered hallmarks of human cancer. Some of the genomic imbalances lead to activation of dominant acting oncogenes, such as *AKT2* and *AKT1*, or the inactivation of recessive acting tumor suppressor genes, such as *NF2* and *CDKN2A/ARF,* which are thought to play a fundamental role in tumorigenesis. A major goal of the research conducted in this laboratory is to understand the biological and clinical implications of recurrent genomic alterations occurring in malignant mesotheliomas (MM), highly aggressive tumors that develop most frequently in the pleura of patients exposed to asbestos fibers. Various molecular approaches are being used to functionally characterize genes contributing to the pathogenesis of MM. Our second major research goal is to understand AKT/protein kinase B function and oncogenic activity. AKT2, one of three members of the AKT family, was originally characterized in this laboratory, and the AKT kinases have been implicated in various cellular responses, including tumor cell survival, proliferation, and invasiveness. AKT activation is one of the most frequent alterations observed in human cancer, and tumor cells with constitutively active AKT may have a dependence on AKT activity for survival. Thus, studies are underway to exploit AKT as a target for therapeutic intervention.

**Molecular biology of malignant mesothelioma (MM).** Altomare, Gallagher, Hoelzle, Pei, Poulikakos, Xiao, Zhang, Testa, in collaboration with Carbone,[c] Chernoff,[§] Gaudino,[d] Jhanwar,[e] Kane,[f] Kruger,[§] Maruta,[g] Mossman,[h] Mutti,[d] Passi[i]

In MMs, deletions of the short (p) arm of chromosome 9 and loss of one copy of chromosome 22 are frequently observed. The deletions of 9p consistently overlap the *CDKN2A/ARF* locus, which encodes the tumor suppressor gene products p16[INK4a] and p14[ARF]. Homozygous deletions of *p16 [INK4a]* and *p14[ARF]* occur in many MMs, and collectively would affect adversely both pRb and p53 growth regulatory pathways, respectively. The neurofibromatosis type 2 tumor suppressor gene (*NF2*) is a critical target of chromosome 22 losses in MM. We have previously identified frequent biallelic inactivation of the *NF2* gene in MMs, which results in loss of expression of the NF2 protein, merlin. We, and others, have also demonstrated that merlin is phosphorylated in response to

expression of activated forms of the Rho GTPases Rac and Cdc42. Merlin is phosphorylated on serine 518, and phosphorylation at this site is mediated by the Pak family of serine/threonine kinases, which are downstream effectors of both Rac and Cdc42. Recently, it has been shown that merlin expression inhibits Rac/Pak activation, which may be attributed to merlin's tumor suppressor function.

In addition to MM, a highly invasive form of cancer characterized by often massive local spreading, inactivation of *NF2* has also been observed in certain benign tumors, particularly Schwannomas, seen in patients with the hereditary disorder known as NF2 syndrome. The mechanism by which merlin acts as a tumor suppressor in both benign and malignant tumors has been obscure. Adenovirus-mediated expression of merlin in NF2-deficient tumor cells was found to inhibit cell proliferation and arrest cells at G1 phase, concomitant with decreased expression of cyclin D1, inhibition of CDK4 activity, and dephosphorylation of pRB. The effect of merlin on cell cycle progression was partially overridden by ectopic expression of cyclin D1. RNA interference experiments showed that silencing of the endogenous *NF2* gene in NF2-positive cells results in upregulation of cyclin D1 and S-phase entry. Furthermore, PAK1-stimulated cyclin D1 promoter activity was repressed by cotransfection of NF2, and PAK activity was inhibited by expression of merlin. Collectively, our data indicate that merlin exerts its antiproliferative effect, at least in part, via repression of Pak-induced cyclin D1 expression, suggesting a unifying mechanism by which merlin inactivation contributes to the tumorigenic growth seen in both noninvasive (benign) and malignant tumors.

To further delineate the significance of *NF2* inactivation in MM and identify tumor suppressor gene alterations that cooperate with *NF2* inactivation in MM pathogenesis, we treated *Nf2* (+/–) knockout mice with asbestos to induce MMs. Asbestos-exposed *Nf2* (+/–) mice exhibited markedly accelerated formation of MMs compared with asbestos-treated wild-type (WT) littermates. Loss of the WT *Nf2* allele, leading to biallelic inactivation, was observed in all nine asbestos-induced MMs from *Nf2* (+/–) mice and in 50% of MMs from asbestos-exposed WT mice. For a detailed comparison with the murine model, DNA analyses were also done on a series of human MM samples. Remarkably, just as in human MMs, tumors from *Nf2* (+/–) mice showed frequent homologous deletions of the *Cdkn2a/Arf* locus and adjacent *Cdkn2b* tumor suppressor gene, as well as reciprocal inactivation of *Tp53* in a subset of tumors that retained the *Arf* locus. As in the human disease counterpart, MMs from the *Nf2* (+/–) mice also showed frequent activation of Akt kinase, which plays a central role in tumorigenesis and therapeutic resistance. This is noteworthy, because MMs are usually diagnosed at an advanced disease stage and are refractory to conventional therapy. Thus, this murine model of environmental carcinogenesis faithfully recapitulates many of the molecular features of human MM and has significant implications for the further characterization of MM pathogenesis and preclinical testing of novel therapeutic modalities.

Although human MMs are generally associated with exposure to asbestos, SV40 virus has been proposed as a possible cofactor in the etiology of some MMs. Although asbestos fibers are known to induce cytotoxicity in human mesothelial cells (HMC), cell survival activated by key signaling pathways may promote transformation. We and others previously reported that SV40 large T antigen induces autocrine loops in HMC and MM cells, leading to activation of growth factor receptors. In recent collaborative work with G. Gaudino and L. Mutti, SV40 was shown to induce cell survival via AKT activation in MM and HMC cells exposed to asbestos. Prolonged exposure to asbestos fibers progressively induced transformation of SV40-positive HMC. As a working model of SV40/asbestos co-carcinogenesis, it was proposed that MM originates from a subpopulation of transformed stem cells and that AKT signaling is a novel therapeutic target to overcome MM resistance to conventional therapies.

As part of Fox Chase Cancer Center's comprehensive initiative on cancer prevention, we are working with a local diagnostics company to validate a new diagnostic kit to detect MM. The objective is to perform validation studies to confirm the merits of the test and to establish an infrastructure for long-term (~10 years) studies in which blood would be collected annually from individuals at high risk of developing MM and tested with the diagnostic kit. Initial work by another group has suggested

that the test has the ability to detect MM at an early stage, when the tumor may be managed surgically and the chances of a cure are greatly improved. A positive test would identify persons who need to undergo extensive medical evaluation to confirm the actual presence of a MM. Such early detection and immediate treatment could result in an improved survival outlook.

**AKT function and oncogenic activity.** Altomare, Knepper,[j] Liu, Mabuchi, Pei, Poulikakos, Rao, Timakhov, Wang, You, Testa, in collaboration with Cheng,[k] Di Cristofano,[§] Godwin,[§] Hamilton,[§] Klein-Szanto,[§] Ozols,[§] Ruggero[§]

Work we reported in the early 1990's showed that *AKT2* is amplified and overexpressed in some ovarian and pancreatic carcinomas. In addition, we demonstrated that *AKT2* antisense RNA can greatly diminish the tumorigenic phenotype of pancreatic cancer cells harboring amplified *AKT*2. We also showed that the AKT2 kinase is often activated in pancreatic cancers, due to upstream perturbations of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, including elevated PI3K activity and/or diminished expression of PTEN phosphatase, the negative regulator of this pathway.

To determine the frequency of AKT activation in human ovarian cancer, we screened a tumor tissue microarray with a phospho-specific pan-AKT (Ser473) antibody, which recognizes the active forms of all three AKT family members (i.e., AKT1, AKT2 and AKT3). Immunohistochemical analysis revealed elevated phospho-AKT staining in 21 of 31 (68%) ovarian carcinomas, which was significantly associated with that of phospho-mTOR (mammalian target of rapamycin), the activated form of a downstream effector of AKT. We next tested the effects of AKT/mTOR activation on the therapeutic sensitivity of ovarian cancer cells. Ovarian cancer cell lines with constitutive activation of AKT, but not cell lines with low basal levels of AKT activity, showed increased cisplatin-induced apoptosis when pre-treated with the PI3K inhibitor LY294002. In addition, treatment with the mTOR inhibitors such as rapamycin and RAD001 resulted in G1 arrest in ovarian cancer cell lines with elevated AKT activity, but not in cell lines with low AKT activity. Collectively, these findings indicate that AKT and mTOR represent potentially impor-

tant therapeutic targets in ovarian cancers exhibiting activation of the AKT signaling pathway.

We have also investigated AKT activation in other human types of human cancer, such as MM. We observed frequent activation (17 of 26, 65%) of AKT in human MM specimens. Consistent with reports implicating hepatocyte growth factor (HGF)/Met receptor signaling in MM, all nine human MM cell lines tested had HGF-inducible AKT activity. One of these cell lines had elevated AKT activity under serum-starvation conditions, which was associated with a homozygous deletion of PTEN. Treatment of this cell line with the mTOR inhibitor rapamycin resulted in growth arrest in G1 phase. Treatment of MM cells with the PI3K inhibitor LY294002 in combination with cis-platin had greater efficacy in inhibiting cell proliferation and inducing apoptosis than either agent alone. Collectively, these data indicate that MMs frequently express elevated AKT activity, which may be targeted pharmacologically to enhance chemotherapeutic efficacy.

Like AKT activation, overexpression of fatty acid synthase (FAS) is frequently observed in human ovarian cancer. To explore a possible connection between AKT and FAS, immunohistochemical analyses were conducted on an ovarian cancer tissue microarray, which revealed a significant correlation between phosphorylated AKT (phospho-AKT) and expression of FAS. To investigate the relationship between phospho-AKT and FAS *in vitro*, a variety of experiments employing the PI3K inhibitor LY294002, inducible PTEN expression in PTEN-null cells, or AKT1 siRNA were used to demonstrate that PI3K/AKT signaling modulates FAS expression. In contrast, inhibition of FAS activity by the drug C75 resulted in down-regulation of phospho-AKT and increased cell death. To explore the functional relationship between phospho-AKT and FAS, we used SKOV3, C200, and OVCAR10 ovarian carcinoma cells, which have constitutively active AKT, and OVCAR5 cells, which have very low basal phospho-AKT levels. Treatment with LY294002 abolished AKT activity and potentiated apoptosis induced by FAS inhibitors cerulenin or C75 only in cells with constitutively active AKT, suggesting that constitutive activation of AKT protects against FAS inhibitor-induced cell death. Furthermore, pharmaco-

logical inhibition of FAS activity resulted in downregulation of phospho-AKT, which preceded the induction of apoptosis. To investigate the relationship between phospho-AKT and FAS *in vivo*, severe combined immunodeficient mice injected intraperitoneally with SKOV3 cells were treated with C75. Growth of SKOV3 xenografts was markedly inhibited by C75. Analysis of the levels of phospho-AKT and FAS in C75-treated tumors revealed concordant downregulation of phospho-AKT and FAS. Collectively, our findings are consistent with a working model in which AKT activation regulates FAS expression, at least in part, whereas FAS activity modulates AKT activation.

AKT is known to promote cell survival through the phosphorylation of several different downstream substrates. To further explore mechanisms by which AKT regulates cell survival, we collaborated with J. Cheng to identify an AKT-interacting protein by yeast two-hybrid screening. This protein is highly homologous to ARG-binding protein 2 (ArgBP2) with splicing of exon 8 of the coding region of ArgBP2. As two splicing isoforms (ArgBP2-alpha and -beta) of ArgBP2 were previously identified, this new isoform was named ArgBP2-gamma. ArgBP2-gamma contains four AKT phosphorylation consensus sites, a SoHo motif, and three Src homology (SH) 3 domains and binds to C-terminal proline-rich motifs of Akt through its first and second SH3 domains. ArgBP2-gamma also interacts with PAK1 via its first and third SH3 domains, suggesting that the SH3 domains of ArgBP2-gamma act as docking sites for AKT and PAK1. AKT was found to phosphorylate ArgBP2-gamma both *in vitro* and *in vivo*. Expression of ArgBP2-gamma was found to induce PAK1 activity and override apoptosis induced by ectopic expression of Bad or DNA damage. Both non-phosphorylatable (ArgBP2-gamma-4A) and SH3 domain-truncated mutant forms of ArgBP2-gamma inhibited AKT-induced PAK1 activation and reduced AKT and PAK1 phosphorylation of Bad and anti-apoptotic function. These findings indicate that ArgBP2-gamma is a physiological substrate of AKT, functions as an adaptor for AKT and PAK1, and plays a role in cell survival pathways regulated by AKT/PAK1.

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