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Glioma Pathogenesis-Related Protein 1: Tumor-Suppressor Activities and Therapeutic Potential

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After glioma pathogenesis-related protein 1 (*GLIPR1/Glipr1*) was identified, the expression of *GLIPR1* was shown to be down-regulated in human prostate cancer, owing in part to methylation in the regulatory region of this gene in prostate cancer cells. Additional studies showed that *GLIPR1/Glipr1* expression is induced by DNA-damaging agents independent of p53. Functional analysis of *GLIPR1* using *in vitro* and *in vivo* gene-transfer approaches revealed both growth suppression and proapoptotic activities for mouse *Glipr1* and human *GLIPR1* in multiple cancer cell lines. The proapoptotic activities were dependent on production of reactive oxygen species and sustained c-Jun-NH_2 kinase signaling. It was interesting that adenoviral vector-mediated *Glipr1* (AdGlipr1) transduction into prostate cancer tissues using an immunocompetent orthotopic mouse model revealed additional biologic activities consistent with tumor-suppressor functions. Significantly reduced tumor-associated angiogenesis and direct suppression of endothelial-cell sprouting activities were documented. In addition, AdGlipr1 strongly stimulated antitumor immune responses that resulted in specific cytotoxic T-lymphocyte activities in this model. *Glipr1*-related antitumor immunostimulatory activities were confirmed and extended in subsequent studies. Administration of a novel *Glipr1* gene-modified tumor cell vaccine had significant antitumor activity in a mouse model of recurrent prostate cancer. In conclusion, restoration of *GLIPR1* function in prostate cancer cells through *GLIPR1* gene-based or GLIPR protein-based delivery methods may provide a safe and effective approach for targeted therapy for a range of malignancies.

Key Words: Glioma pathogenesis-related protein 1, tumor suppressor, prostate cancer

INTRODUCTION

Glioma pathogenesis-related protein 1 (*GLIPR1/Glipr1*) is a member of the cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins (CAP) superfamily.[1] Early studies showed that *GLIPR1* was associated with myelomonocytic differentiation toward the macrophage phenotype.[2] *GLIPR1* was later identified as a p53 target gene and was shown to be methylated and down-regulated in prostate cancer.[3,4] Functional analysis of *GLIPR1* using *in vitro* and *in vivo* gene-transfer approaches revealed both growth suppression and proapoptotic activities for mouse *Glipr1* and human *GLIPR1* in multiple cancer cell lines.[3-6] The proapoptotic activities were shown to depend on production of reactive oxygen species

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(ROS) and sustained c-Jun-NH_2 kinase (JNK) signaling.[5]

Further study showed that mice with an inactivated *Glipr1* gene had significantly shorter tumor-free survival times than did either *Glipr1*$^{+/+}$ or *Glipr1*$^{+/-}$ mice in both *p53*$^{+/+}$ and *p53*$^{+/-}$ genetic backgrounds owing to the development of a unique array of malignancies.[5] It was interesting that adenoviral vector-mediated *Glipr1* (AdGlipr1) transduction into prostate cancer tissues using an immunocompetent orthotopic mouse model revealed additional biologic activities consistent with tumor-suppressor functions. Significantly reduced tumor-associated angiogenesis and direct suppression of endothelial-cell sprouting activities were documented.[6] In addition, AdGlipr1 strongly stimulated antitumor immune responses that resulted in specific cytotoxic T-cell (CTL) activities in this model. *Glipr1*-related antitumor immunostimulatory activities were confirmed and extended in subsequent studies. Administration of a novel *Glipr1* gene-modified tumor cell vaccine had significant antitumor activity in a mouse model of recurrent prostate cancer.[7]

These preclinical study and other results led to the initiation and completion of a clinical trial in which an adenoviral vector-mediated *GLIPR1* neoadjuvant injection was tested in men with high-risk prostate cancer preceding radical prostatectomy.[8] Additional AdGLIPR1 clinical testing and potential development of *GLIPR1* protein-based therapies are under consideration.

The CAP superfamily and GLIPR1 subfamily

The CAP superfamily was named after it was recognized that considerable sequence similarity exists among the cysteine-rich secretory proteins (CRISPs), antigen 5, and pathogenesis-related proteins.[1] CRISPs are highly enriched in the male mammalian reproductive tract and in the venom-secretory ducts of snakes, lizards, and other vertebrates.[9,10] The highly immunogenic antigen 5 proteins are abundant in the venom-secretory ducts of stinging insects,[11] and the pathogenesis-related 1 proteins are up-regulated in plants after invasion by pathogens.[12] Speculation about the functional relationships among these CAP proteins has led to the idea that they may actually be isozymes with distinct substrate specificity[13] and overlap between the plant and human immune systems.[14]

The *GLIPR1* gene was initially identified as being up-regulated within glioblastoma multiforme and astrocytoma tissues and in glioma cell lines.[15] Shortly after publication of that initial report, a subsequent study identified the same gene in glioma cell lines and called it "related to testes-specific, vespid, and pathogenesis protein 1 (RTVP-1)".[16] We also identified *Rtvp-1/Glipr1* in a differential-display polymerase chain reaction (PCR) screen as a p53 target gene.[3] Identification of *GLIPR1/Glipr1* was followed by identification and cloning of multiple isoforms of additional *GLIPR1* subfamily genes. Mammalian *GLIPR1* proteins are a multigene subfamily that consists of three genes in most species and four genes in the mouse.[1] We previously identified two human *GLIPR1*-like genes (*GLIPR1L1* and *GLIPR1L2*) and three mouse *Glipr1*-like genes (*Glipr1l1, Glipr1l2,* and *Glipr1l3*) as members of the *GLIPR1* subfamily.[1,17] An important note is that human *GLIPR1, GLIPR1L1*, and *GLIPR1L2* genes are closely clustered on human chromosome 12q21 and mouse *Glipr1, Glipr1l1, Glipr1l2*, and *Glipr1l3* genes, onmouse chromosome 10D1.[17] In addition, we found that all three members of the human *GLIPR1* cluster are direct p53 targets. It is also important that we further identified and characterized multiple alternative transcripts for *GLIPR1L1* and *GLIPR1L2*.[17] The presence of a putative signal peptide sequence and extracellular protein signature motifs suggests that most of the *GLIPR1*-cluster proteins are located on the surface of the cell membrane or secreted. Many, but not all *GLIPR1*-cluster proteins also contain a transmembrane domain, suggesting different capacities for secretion.[17] These results indicate that important regulatory functions are encoded in the *GLIPR1/Glipr1* subfamily proteins.

GLIPR1 subfamily gene expression is also highly tissue specific. For example, in somewhat limited studies, we showed that *GLIPR1* expression is relatively widespread, whereas *GLIPR1L1* expression is highly tissue specific, with very high mRNA levels in the testes but only trace amounts in the bladder and undetectable expression in the prostate, kidney, lung, and bone marrow. *GLIPR1L2*, like *GLIPR1L1*, is highly expressed in the testes, yet relatively low but detectable levels of expression were documented in the prostate, kidney, bladder, lung, and bone marrow.[17]

GLIPR1/Glipr1 as a tumor-suppressor protein

Initial studies of *GLIPR1* expression provided clear evidence of the potential for tumor-suppressor activities. Quantitative reverse-transcriptase PCR and/or in situ hybridization analysis showed that *GLIPR1* expression was lower in primary prostate cancer cells than in normal prostatic epithelium.[4] In addition, prostate cancer cells that were metastatic to lymph nodes demonstrated much lower levels of expression than did normal prostate epithelium or prostate cancer. Although *GLIPR1* mRNA was predominantly localized in prostatic epithelial cells (among which the basal cells exhibited the strongest signal level), some isolated stromal cells also showed moderate *GLIPR1* mRNA levels.

Further, immunostaining analysis of normal prostate, primary prostate cancer, and metastatic prostate cancer samples showed that *GLIPR1* protein expression is significantly lower in primary prostate cancer than in normal prostatic epithelium; *GLIPR1* protein levels are still lower,

or even undetectable, in lymph node metastases.[4] The same study also showed that the human *GLIPR1* promoter is extensively methylated in prostate cancer tissues relative to its methylation in normal prostate and that such increased methylation correlates with decreased levels of *GLIPR1* expression. These data led to the proposal that *GLIPR1* is a tumor suppressor that undergoes epigenetic inactivation in prostate cancer.[4]

Gene-transfer approaches were initially used to demonstrate activities consistent with tumor-suppressor functions of *GLIPR1/Glipr1*. P53-dependent and -independent proapoptotic activities were demonstrated as a result of *GLIPR1/Glipr1* overexpression in multiple prostate cancer cells and various malignant cell lines.[3] It was of interest that the proapoptotic effect was considerably less in nontransformed mouse embryo fibroblasts than in malignant cell lines. That same study also revealed that gamma irradiation and doxorubicin induced substantial levels of *GLIPR1* mRNA in both the presence and absence of p53, which is suggestive of p53-independent *GLIPR1* tumor-suppressor functions. Moreover, we found that deletion of the *GLIPR1/Glipr1* signal peptide significantly reduced the proapoptotic effects of *GLIPR1/Glipr1 in vitro*, suggesting that secreted and/or release or cleavage from the membrane is important for its biologic functions.[3]

These initial studies were extended in subsequent studies that showed that *GLIPR1* overexpression led to significant suppression of colony growth and induction of apoptosis in multiple cancer cell lines.[5] To test our tumor-suppressor hypothesis *in vivo*, we generated mice with an inactivated *Glipr1* gene; these *Glipr1*$^{-/-}$ mice had significantly shorter tumor-free survival times than either *Glipr1*$^{+/+}$ or *Glipr1*$^{+/-}$ mice did in both p53$^{+/+}$ and *p53*$^{+/-}$ genetic backgrounds.[5] An interesting finding was that a wide spectrum of tumors developed in the *Glipr1*$^{-/-}$ mice, including lung carcinomas and plasma cytomas. It was also notable that the progressive loss of *Glipr1* in the *p53*$^{+/-}$ genetic background resulted in progressive reduction of *p53* loss of heterozygosity. These data supported previous *in vitro* data[3,4] and showed that *Glipr1* has independent tumor-suppressor activities under these conditions.

GLIPR1-mediated proapoptotic signaling

As a member of the CAP family, *GLIPR1* contains 11 cysteines that are somewhat concentrated at the carboxyl terminal of the molecule.[17] Because cysteine residues within polypeptides can play important roles in redox homeostasis in mammalian cells,[18,19] we hypothesized that *GLIPR1* overexpression affects cellular ROS generation. The results of extensive analysis showed that *GLIPR1* overexpression led to significantly increased ROS in various tumor cell lines, including prostate cancer cells.[5] Additional studies showed that sustained JNK signaling resulted from *GLIPR1*-stimulated ROS production. Overall, increased ROS generation is required for *GLIPR1*-mediated activation of JNK and ultimately the induction of apoptosis in an inducible bladder-cancer cell model *in vitro*.[5]

These results provided mechanistic underpinning to the notion that *GLIPR1* is a novel broad-spectrum tumor suppressor whose proapoptotic properties are exerted in part through ROS-JNK signaling.

GLIPR1-mediated effects on the tumor microenvironment

As a secreted and/or membrane-bound proapoptotic tumor-suppressor protein, *GLIPR1* (and potentially other *GLIPR1* subfamily proteins) may have unique properties. *GLIPR1* contains both an amino-terminal signal peptide and a transmembrane domain.[17] In addition, the results of our previous studies showed that deletion of the *GLIPR1/Glipr1* signal peptide significantly reduced the proapoptotic effects of *GLIPR1/Glipr1 in vitro*.[3] Thus, it is likely that *GLIPR1* is secreted and/or tethered onto the membranes of cells that express substantial levels of *GLIPR1*. Membrane-bound *GLIPR1* may also undergo proteolytic cleavage, adding to the extracellular pool of *GLIPR1*. Although it is speculative, this biologic scenario would involve a pool of extracellular *GLIPR1* with the potential for significant autocrine and/or paracrine activities.

To move beyond speculation, it will be necessary to directly test *GLIPR1* protein under various conditions using relevant cell types that are present in the prostate cancer microenvironment. However, the results of previous studies are consistent with *GLIPR1*-mediated, multi-cell type-specific tumor-suppressor activities. We previously showed that AdGlipr1 treatment of orthotopic mouse prostate cancer resulted in reduced microvessel density and that AdGlipr1 also directly inhibited endothelial-cell sprouting in a rat aortic-ring sprouting assay.[6] These data are consistent with antiangiogenic activities of secreted and/or membrane-bound GLIPR1/Glipr1 *in vivo*. In addition, the results of previous studies have shown that increased expression of *Glipr1* is associated with macrophage differentiation.[2] Both of these cell types are dominant, active components of the tumor microenvironment.[20] In further support of the notion that secreted or cleaved GLIPR1/Glipr1, we have shown that a vaccine prepared with mouse prostate cancer cells, which were transduced with *Glipr1* and irradiated, significantly reduced orthotopic prostate cancer "tumor take" and establishment of experimental prostate cancer lung metastases.[7] Increased natural-killer cell and CTL activities were documented in those studies, suggesting direct systemic immunostimulatory activities. Overall, these data suggest that prostate tumor cell-derived

secreted or cleaved GLIPR1/Glipr1 may both exert direct proapoptotic antitumor effects and suppress tumorigenesis and/or local tumor growth through antiangiogenic and immunostimulatory effects within the prostate cancer microenvironment. Further studies are required to confirm this hypothesis.

GLIPR1 as a therapeutic agent

Elucidation of the unique tumor-suppressor properties of *GLIPR1/Glipr1* led to the notion that *GLIPR1* treatment may be effective for local and/or systemic control of prostate cancer and, potentially, other malignancies. Our preclinical studies, described above, showed that direct injection of AdGlipr1 into prostate cancer tissues using an immunocompetent orthotopic mouse model resulted in significant tumor growth suppression and longer survival of tumor-bearing animals.[6] Analysis of AdGlipr1-treated tissues demonstrated increased prostate cancer cell apoptosis and significantly reduced tumor-associated angiogenesis. In addition, AdGlipr1-stimulated antitumor immune responses resulted in specific CTL activities in this model. *Glipr1*-related antitumor immunostimulatory activities were confirmed and extended in subsequent studies. Administration of a novel *Glipr1* gene-modified tumor cell vaccine to mice had significant antitumor activity in a preclinical model of recurrent prostate cancer.[7]

On the basis of the results of our basic and preclinical studies, we completed a phase 1b clinical trial of in situ, adenoviral vector-mediated, neoadjuvant, pre-radical prostatectomy *GLIPR1* gene therapy in patients with locally advanced adenocarcinoma of the prostate (IND13033). Preliminary analysis of the data revealed that intraprostatic administration of AdGLIPR1 was safe in men with localized high-risk prostate cancer before radical prostatectomy. In addition, preliminary evidence of biologic antitumor, systemic, and local activity was observed, suggesting a role for further development in the perioperative setting.[8]

Because the growth-arrest and proapoptotic effects of *GLIPR1* are likely mediated to a large extent through autocrine and/or paracrine activities in prostate cancer, we have undertaken the development of GLIPR1 protein-based anticancer therapy. It is conceivable that GLIPR1 protein therapy would have important advantages compared with viral vector-based *GLIPR1* delivery under specific conditions. For example, GLIPR1 protein may be delivered systemically and therefore be potentially effective against metastatic disease.

SUMMARY

We have identified and characterized GLIPR1/Glipr1 as a secreted tumor-suppressor protein. *GLIPR1/Glipr1* is a member of the *GLIPR1* subfamily that includes multiple proteins with potentially unique but overlapping functions. *GLIPR1/Glipr1* is regulated by p53 yet demonstrates p53-independent tumor-suppressor activities. *GLIPR1/Glipr1* can suppress the growth of multiple tumor cells and has potent proapoptotic activities both *in vitro* and *in vivo*. *GLIPR1* overexpression stimulates proapoptotic activities through sustained ROS-JNK signaling. Of note, *GLIPR1/Glipr1* can also suppress angiogenesis and possesses immunostimulatory properties in vivo. Thus, in addition to direct tumor cell-specific growth arrest and proapoptotic activities, the tumor-suppressor activities of *GLIPR1/Glipr1* may involve the tumor microenvironment. Identification of *GLIPR1/Glipr1* and *GLIPR1* subfamily members provides an opportunity for development of *GLIPR1*-based gene and protein therapies for prostate cancer and other malignancies.

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