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***mda-7/IL-24*: A Unique Member of the IL-10 Gene Family Promoting Cancer-Targeted Toxicity**

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Abstract

Melanoma differentiation associated gene-7/interleukin-24 (*mda-7/IL-24*) is a unique member of the IL-10 gene family that displays nearly ubiquitous cancer-specific toxicity, with no harmful effects toward normal cells or tissues. *mda-7/IL-24* was cloned from human melanoma cells by differentiation induction subtraction hybridization (DISH) and promotes endoplasmic reticulum (ER) stress culminating in apoptosis or toxic autophagy in a broad-spectrum of human cancers, when assayed in cell culture, *in vivo* in human tumor xenograft mouse models and in a Phase I clinical trial in patients with advanced cancers. This therapeutically active cytokine also induces indirect anti-tumor activity through inhibition of angiogenesis, stimulation of an anti-tumor

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immune response, and sensitization of cancer cells to radiation-, chemotherapy- and antibody-induced killing.

Keywords

mda-7/IL-24; apoptosis; autophagy; bystander antitumor activity; cancer terminator virus

1: Introduction and Overview

Cancer is a progressive disease with advanced tumors frequently displaying resistance to multiple therapies, including chemotherapy, immunotherapy and radiation (1–3). Despite intensive investigation, solid and hematopoietic tumors remain formidable clinical challenges resulting in high frequencies of morbidity and mortality (2, 3). Reprogramming tumor cells to undergo programmed cell death (apoptosis) represents a promising and powerful strategy for treating both local and metastatic disease (4–10). Approaches to achieve this objective involve replacement of defective tumor suppressor genes and/or expression/activation of apoptosis-inducing and/or toxic autophagy-inducing genes or their products in tumor cells (4–6, 11). This aspiration is frequently achieved using small molecule drugs or using viruses, such as adenoviruses (Ads), to deliver tumor suppressor or toxic gene products.

Melanoma differentiation associated gene-7 (*mda-7*) was discovered in our laboratory by subtraction hybridization of temporally-spaced subtracted cDNA libraries prepared from terminally differentiated human melanoma cells treated with human fibroblast interferon (IFN- β) and the protein kinase C activator mezerein (MEZ), an approach called ‘*differentiation induction subtraction hybridization*’ (DISH) (12–16). *mda-7* is located in human chromosome 1q32–33 and based on sequence homology, chromosomal localization, and its functional properties, the *mda-7* gene is now classified as a member of the IL-10 family of cytokines and named IL-24 (17, 18). The *mda-7/IL-24* cDNA encodes a protein of 206-amino acids with a predicted size of ~24-kDa, which contains an interleukin (IL)-10 signature motif at amino acids 101–121 (SDAESCYLVTLLLEFYLYKTVF) shared by other members of the IL-10 family of cytokines (18, 19). Sequence analysis revealed the presence of a 49-amino acid signal peptide suggesting that the molecule could be cleaved and secreted (18). Expression of MDA-7/IL-24 protein was detected in cells of the immune system (mainly by expression in tissues associated with the immune system, such as spleen, thymus and PBMC) and normal human melanocytes (14, 17, 20). Of interest, a progressive loss of MDA-7/IL-24 expression during melanoma progression suggests an inverse relationship between MDA-7/IL-24 expression and the evolution of melanocytes to various stages of melanoma (14, 21).

Jiang et al. (14, 22) first demonstrated the growth suppressive properties of *mda-7/IL-24* in human melanoma and other cancer cells. Subsequent studies provided consistent evidence that ectopic expression of *mda-7/IL-24* employing a replication incompetent adenovirus (Ad.*mda-7*) resulted in apoptosis induction and cell death in a wide variety of solid tumors including melanoma (23–28), malignant glioma (29–38), carcinomas of the breast (39–43), kidney (44, 45), cervix (17), colorectum (46), liver (47–51), lung (52–60), ovary (61–65) and prostate (66–76) (Table-1) sparing normal cellular counterparts, i.e., such as normal melanocytes, astrocytes, fibroblasts, and mesothelial and epithelial cells (Table 1) (23, 24, 29, 56, 73, 77–79). The *in vitro* antitumor activity of *mda-7/IL-24* readily translated into the *in vivo* situation in animal models containing human breast, prostate, lung and colorectal carcinomas and in malignant glioma xenografts (31, 42, 53, 56). Moreover, the ability of *mda-7/IL-24* to induce a potent “bystander cancer-specific killing effect” (67, 78) provides

an unprecedented opportunity to use this molecule to target for destruction not only primary tumors, but also metastases. Based on its profound cancer-selective tropism, substantiated by *in vivo* human xenograft studies in nude mice, *mda-7/IL-24* (administered as *Ad.mda-7*) was evaluated in a phase I clinical trial in patients with melanomas and solid cancers (80–82). These studies document that *mda-7/IL-24* is well tolerated and demonstrates evidence of significant (~44%) clinical activity (82). This review focuses on the recent enhancements in our understanding of the mode of action of *mda-7/IL-24* and its potential applications as a unique and promising effective cytokine-based gene therapy for human cancers.

2: *mda-7/IL-24* induces ubiquitous antitumor effects through modulation of diverse processes and signaling pathways

2.1.1: Induction of cancer-specific apoptosis by *mda-7/IL-24*

The molecular basis of *mda-7/IL-24*-mediated cancer-specific induction of apoptosis (Figure 1) has been extensively scrutinized (7, 83–86). Infection of prostate cancer cells with *Ad.mda-7* causes endoplasmic stress (ER stress) by physically interacting with the ER chaperon protein BiP/GRP78, which culminates in induction of a cascade of Unfolded Protein Response (UPR) (54, 79). The ER stress-mediated cancer cell toxicity by *mda-7/IL-24* is further supported by studies by Pataer et al. where an adenovirus was designed to target MDA-7/IL-24 expression to the ER (*Ad.ER-mda-7*), which resulted in enhanced killing in lung and esophageal cancer cells as compared to infection with *Ad.mda-7* (87). Despite the presence of MDA-7/IL-24 protein in both normal and cancer cells, the ER-stress response-induced biochemical and apoptotic changes were observed only in cancer cells of diverse tissue origin and not in their normal cellular counterparts. The level of BiP/GRP78 is higher in multiple types of cancer cells than in normal cells, which might explain why cancer cells are more susceptible to ER stress by *mda-7/IL-24* (88). In melanoma cells, the *mda-7/IL-24*-mediated UPR leads to activation of p38 MAPK activity and the induction of growth arrest and DNA damage inducible (GADD) genes that culminate in apoptosis (24). The inhibition of p38 MAPK activity by either pharmacological or genetic inhibitors results in development of resistance to *mda-7/IL-24*-mediated cytotoxicity. Similarly, inhibition of the GADD family of genes by an antisense approach protects cells from *Ad.mda-7*-mediated cell death. Furthermore, it is reported that p38 MAPK regulates the expression of *mda-7/IL-24* by stabilization of the 3' UTR of IL-24 mRNA (89, 90).

Recently, the importance of ceramide in inducing cell death by *mda-7/IL-24* has been documented in multiple types of cancers (45, 68, 91). Sauane et al. demonstrated that *Ad.mda-7* infection of tumor cells, but not normal cells, resulted in increased ceramide accumulation (68). Infection with *Ad.mda-7* induced a marked increase in various ceramides (C16, C24, C24:1) selectively in prostate cancer cells. Inhibiting the enzyme serine palmitoyltransferase (SPT) using the potent SPT inhibitor myriocin (ISP-1), impaired *mda-7/IL-24*-induced apoptosis and ceramide production, suggesting that ceramide formation caused by *Ad.mda-7* occurs through *de novo* synthesis. Pretreatment of cells with Fumonisin B1 (FB1) or ISP-1 abolished the induction of ER stress markers (BiP/GRP78, GADD153 and phospho-eIF2 α) triggered by *Ad.mda-7* infection indicating that ceramide mediates ER stress induction by *Ad.mda-7*. Additionally, *Ad.mda-7* activated protein phosphatase 2A (PP2A) and promoted dephosphorylation of the anti-apoptotic molecule BCL-2, a downstream ceramide-mediated pathway of *mda-7/IL-24* action (68). In kidney cancer cells, malignant glioma and ovarian cancer cells, *mda-7/IL-24* (*Ad.mda-7* as well as GST-MDA-7 (a GST fusion recombinant form of *mda-7/IL-24*) activated CD95 in a manner that is dependent on the actions of multiple enzymes or pathways that generate ceramide, which promote PERK-dependent cell death and autophagy (45, 64, 91).

In specific non-small cell lung cancer (NSCLC) cells, *mda-7/IL-24* induces apoptosis by activating the up regulation of double-stranded RNA-dependent protein kinase (PKR) (60, 92). Interestingly, it is documented that PKR interacts physically with MDA-7/IL-24. Activation of PKR by *mda-7/IL-24* results in activation of its downstream targets, i.e., eIF2 α , Tyk2 and p38 MAPK, resulting in inhibition of global protein synthesis. Treatment of NSCLC cells with 2-amino purine, a pharmacological inhibitor of serine/threonine kinase blocked Ad.*mda-7*-mediated apoptosis as well as activation of PKR (92, 93). Additionally, mouse embryonic fibroblast cells derived from PKR-null mice showed resistance towards *mda-7/IL-24*-mediated cytotoxicity. In melanoma cells, p38 MAPK activation is downstream of PKR activation, although in melanoma cells the post-p38 signal transduction changes appear to be more important in Ad.*mda-7*-induced apoptosis (24). It has been documented that eIF2 α phosphorylation activates the transcription factor ATF4, which activates GADD153 (94). Thus, there is a significant level of cross talk between the PKR and the p38 MAPK signal transduction pathways.

The involvement of c-Jun NH2-terminal kinase (JNK) pathway in *mda-7/IL-24*-mediated apoptosis in specific cancer cells makes them amenable to enhanced toxicity through synergy with irradiation (95). Infection with Ad.*mda-7* radiosensitizes malignant glioma and prostate cancer cells facilitating the induction of tumor cell death (29, 32, 66). A combination treatment of Ad.*mda-7* or GST-MDA-7 and γ -irradiation activates JNK and treatment with a specific JNK inhibitor, SP600125, protected tumor cells from the synergistic killing effect of radiation and Ad.*mda-7*. Additionally, in NSCLC, curcumin, a dietary pigment that inhibits JNK activation, inhibited phosphorylation of c-jun and radiosensitization by Ad.*mda-7* (95).

Bcl-2 family members are frequently implicated in the acquisition of resistance to apoptosis by malignant cancer cells (96). In several contexts, various therapeutic treatments result in preferential down regulation of anti-apoptotic protein and up regulation of pro-apoptotic proteins. *mda-7/IL-24* has been shown to inhibit anti-apoptotic genes and activate tumor suppressor and pro-apoptotic genes. In breast carcinoma cells, Ad.*mda-7* alters the ratio of specific pro-apoptotic (Bax, Bad, Bak and Bcl-xS) to anti-apoptotic (Bcl-2, Bcl-xL and Bcl-W and Mcl-1) proteins tilting the balance from survival to death (42, 97). It should be noted that Ad.*mda-7* could induce apoptosis in *bax*-null DU-145 prostate cancer cells (25); therefore, a *bax*-independent pathway can also mediate apoptosis. Our ongoing studies suggest that the pro-apoptotic protein Bak plays a critical role in regulating cell death by profound Mcl-1 down regulation in ovarian (64) and prostate cancer cells (73).

Another mechanism conferring cancer specificity to *mda-7/IL-24* involves oxidative stress by generation of reactive oxygen species (ROS) followed by mitochondrial dysfunction uniquely in cancer cells (98–100). In prostate cancer cells, *mda-7/IL-24* induces apoptosis in a ROS-dependent manner, which was inhibited in the presence of antioxidants and mitochondria permeability inhibitors. Recently, in GBM cells it has been shown that Ad.*mda-7* infection increases expression of superoxide dismutase 2 (SOD2) and thioredoxin (TRX) and by 24 h increased eEF2 phosphorylation (91). In primary GBM cells, quenching of ROS production by over-expression of SOD2 or TRX suppresses autophagy, activation of JNK1–3 and cell killing, whereas inhibition of SOD2 or TRX enhances autophagy, cell death and activation of JNK1–3 and p38 MAPK, and enhanced inactivation of ERK1/2 and AKT. Support for this hypothesis comes from a previous study by Emdad et al. study showing that multidrug resistant colorectal cancer cells with high basal ROS levels were more susceptible to Ad.*mda-7*-induced cell death than its non-drug resistant counterpart that contained relatively low levels of basal ROS (46). This study indicated that ROS-inducing agents can overcome natural anti-oxidants much more efficiently in cancer cells, which generally have enhanced basal ROS levels, as opposed to normal cells, thus inducing

apoptosis and cell death selectively in the tumor cells. Furthermore, microarray analysis demonstrated increased expression of the tumor suppressor genes E-cadherin, APC, GSK-3 β and PTEN and decreased expression of proto-oncogenes involved in β -catenin and PI3K signaling following infection with Ad.mda-7 in breast and lung cancer cells (101).

In addition to the potent cytotoxic effect, the ubiquitous antitumor effect of mda-7/IL-24 *in vivo* is also mediated by its ability to inhibit angiogenesis (7, 72, 102), invasion and migration of cancer cells (55). Ad.mda-7 displayed cytotoxicity *in vitro* toward a panel of human lung tumor cells, but not to endothelial cells *in vitro* (52). *In vivo* molecular analysis of the growth inhibitory effects of mda-7/IL-24 toward tumor cells demonstrated that Ad.mda-7-treated tumors had reduced tumor vascularization compared to control vector-treated tumors. These results suggested that the reduced tumor vascularization induced by Ad.mda-7 in treated tumors could be due to direct tumor killing or anti-angiogenic activity. To test whether mda-7/IL-24-mediated an anti-angiogenic activity, *in vitro* and *in vivo* experiments were conducted (7). In *in vitro* studies, Ad.mda-7-treated human H1299 lung tumor cells that served as an ectopic source for MDA-7/IL-24 protein were mixed with human umbilical vein endothelial cells (HUVEC) and plated onto Matrigel coated 96-well plates and observed for endothelial cell differentiation (ECD), an assay that is routinely used to test for anti-angiogenic activity. Tumor cells that were treated with PBS or infected with Ad.luc (vector control) and mixed with HUVEC served as controls. A marked inhibition of ECD was observed in wells containing Ad.mda-7-treated tumor cells (7), whereas no inhibition of ECD was observed in Ad.luc. Similarly, MDA-7/IL-24 protein selectively inhibited ECD with no effect on cell proliferation by inhibiting vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). In addition, involvement of IL-22R in MDA-7/IL-24 protein-mediated anti-angiogenic activity was confirmed by activation of STAT-3 in HUVEC as a measure of receptor-ligand interaction and by receptor-blocking studies (102).

2.1.2: Role of autophagy and apoptosis induction by mda-7/IL-24 in cancer cell toxicity

Apart from the cancer-specific apoptotic potential, MDA-7/IL-24 also plays an important role in autophagy, which regulates both cancer cell survival and programmed cell death. Autophagy is a dynamic process in which intracellular membrane structures sequester proteins and organelles to degrade and turn over these materials. In addition to its basic role in the turnover of proteins and organelles, autophagy has multiple physiological and pathophysiological functions, such as promotion of cancer (103–105). Our understanding of the role of MDA-7/IL-24 on autophagy in cancer is at a very early stage, and even the most fundamental question- whether MDA-7/IL-24 induced autophagy kills cancer cells or protects them from unfavorable conditions requires clarification. This section covers the accumulating data about the role of MDA-7/IL-24-induced autophagy in cancer, and discusses the attractive prospect of manipulating autophagic processes as a new method of cancer therapy.

Recent studies have shown that GST-MDA-7/IL-24 induces a toxic form of autophagy, referred to as type II programmed cell death in glioblastoma multiforme (GBM) cells and transformed fibroblasts (30, 37). GST-MDA-7/IL-24 killed glioma cells with diverse genetic characteristics that correlated with inactivation of ERK1/2 and activation of JNK1–3. Activation of JNK1–3 was dependent on protein kinase R-like endoplasmic reticulum kinase (PERK). PERK activation is generally considered a marker for ER stress and the UPR. PERK activation stimulated the vesicularization of a transfected LC3-GFP protein that correlated with processing of endogenous LC3 (ATG8) and increased expression of other autophagy regulatory proteins such as ATG5 and Beclin1 (30, 37). Additionally, in kidney cancer cells GST-MDA-7 activated CD95 in a manner that is dependent on the actions of multiple enzymes or pathways that generate ceramide, which promote PERK-dependent cell

killing and autophagy. This study discovered that ER stress and UPR induced by MDA-7/IL-24 could be a potent inducer of autophagic cell death pathways (45). In another study, it was shown that MDA-7/IL-24 enhanced the survival of chronic lymphocytic leukemia B-cells and inhibition of autophagy induced by *mda-7/IL-24* strongly potentiated antileukemia activity *in vitro* and *in vivo*. The enforced expression of ZD55-IL-24, a conditionally replicating adenovirus (CRAd) carrying *mda-7/IL-24*-induced autophagy, which was triggered by the up regulation of Beclin-1, and the inhibition of autophagy by phosphatidylinositol 3-kinase inhibitor, wortmanin, caused enhanced cell death *in vitro*, suggesting that the autophagy might protect leukemia cells from the *mda-7/IL-24*-induced apoptosis. Importantly, the combination of *mda-7/IL-24* with wortmanin elicited a strongly enhanced antileukemia efficacy in established leukemia xenografts. These results suggest that *mda-7/IL-24*-induced autophagy in leukemia cells may provide survival advantage and *mda-7/IL-24* combined with agents that disrupt autophagy is a promising new strategy for the treatment of leukemia (106).

The role MDA-7/IL-24-induced autophagy in relation to the therapeutic cancer-specific growth inhibitory potential of MDA-7/IL-24 has been investigated in prostate cancer cells (75). Ad-*mda-7*-induced ER stress and ceramide generation resulted in induction of autophagy in prostate cancer cells, without affecting normal human prostate epithelial cells, via the canonical autophagic pathway, involving Beclin-1, atg5 and hVps34. Autophagy was evident until 24 h post-Ad.*mda-7*-infection and then switched to apoptosis at 48 h. Inhibition of autophagy by 3-methyladenosine (3-MA) significantly increased Ad.*mda-7*-induced apoptosis suggesting that autophagy might first be initiated as a cytoprotective mechanism in prostate cancer cells. Inhibition of apoptosis by over-expression of anti-apoptotic proteins Bcl-2 or Bcl-x_L increased autophagy upon Ad.*mda-7* infection. During the apoptotic phase, MDA-7/IL-24 protein physically interacted with Beclin-1 and this interaction might inhibit Beclin-1 function culminating in apoptosis. Conversely, Ad.*mda-7* infection lead to calpain-mediated cleavage of ATG5 protein that might also facilitate a biochemical switch from autophagy to apoptosis. This study revealed novel aspects of interplay between autophagy and apoptosis that underlie the cytotoxic action of *mda-7/IL-24* in prostate cancer cells. These new insights into *mda-7/IL-24* action provide interesting leads for developing innovative combinatorial approaches for prostate cancer therapy (75).

Studies have been performed to determine if inhibition of multiple cytoprotective cell signaling pathways could enhance the toxicity of recombinant adenoviral delivery of *mda-7/IL-24* toward invasive primary human GBM cells (107). The toxicity of a serotype 5 recombinant adenovirus expressing MDA-7/IL-24 (Ad.5-*mda-7*) was enhanced by combined molecular or small molecule inhibition of MEK1/2 and PI3K or AKT; inhibition of mTOR and MEK1/2; and by the HSP90 inhibitor 17AAG. Molecular inhibition of mTOR / PI3K / MEK1 signaling *in vivo* also enhanced Ad.5-*mda-7* toxicity. In GBM cells of diverse genetic backgrounds, inhibition of cytoprotective cell signaling pathways enhanced MDA-7/IL-24-induced autophagy, mitochondrial dysfunction and tumor cell death. A future combination of these inhibitors with *mda-7/IL-24* holds promise for developing a potentially effective therapy for GBM.

2.1.3: Development of antitumor immune response by *mda-7/IL-24*

Cancer is a progressive disease, which can recur after surgery or initial failed therapy. Treatment is also frequently limited by the ability of cancer cells to metastasize at the time of first diagnosis or after attempted therapeutic intervention. In these contexts, a systemic, specific and sustained immune response against cancer at the time of initial therapy could address the most critical issues: prevention of tumor recurrence and metastasis (108). In most cases, simply killing tumor cells by molecular or tumor-targeted treatments may not be sufficient to raise effective antitumor immunity. To establish the proof-of-principle, the

therapeutic efficacy of intratumoral delivery of a nonreplicating adenoviral vector encoding *mda-7/IL-24* (Ad.*mda-7*) and a secretable form of endoplasmic reticulum resident chaperone grp170 (Ad.sgrp170), a potent immunostimulatory adjuvant and antigen carrier, has been evaluated in prostate cancer (109, 110). Intratumoral administration of Ad.*mda-7* in combination with Ad.sgrp170 was more effective in controlling growth of TRAMP-C2 prostate tumors compared with either Ad.*mda-7* or Ad.sgrp170 treatment. Generation of systemic antitumor immunity was shown by enhanced protection against subsequent tumor challenge and improved control of distant tumors (110). The combined treatments enhanced antigen and tumor-specific T-cell response, as indicated by increased IFN- γ production and cytolytic activity. Similarly, when immune potentiation activity of Ad.*mda-7* in a cancer vaccine model was evaluated, it resulted in a significant increase in the CD3 (+) CD8 (+), but not the CD3 (+) CD4 (+) cell populations. Thus, Ad.*mda-7* treatment of syngeneic tumors induces tumor cell death and promotes immune activation, leading to anticancer immunity (111). Evidence of immune activation following Ad.*mda-7* was demonstrated by increased production of serum inflammatory markers from a Phase I clinical trial of Ad.*mda-7* (INGN-241) in melanoma, which will be discussed later in this review (80–82), where transient increases in circulating cytokines such as IL-6, IL-10 and TNF α in response to *mda-7/IL-24* were observed. Patients receiving intermediate- or high-dose injections showed a significant increase in CD3+CD8+ T cells, at day 15 following injection suggesting that Ad.*mda-7* may be associated with a TH1-like response.

2.1.4: Synergistic Effects of *mda-7/IL-24* with other anticancer agents: overcoming chemotherapy resistance in multiple cancers

The diverse and multiple anticancer signaling mechanisms modified by *mda-7/IL-24* also enable it to synergize with other known anticancer agents including radiation, monoclonal antibodies, and chemotherapeutic agents (such as geldanamycin, vitamin E succinate, sulindac and celecoxib) (35, 58, 59, 63, 66, 112–114). The combination of Ad.*mda-7* and Herceptin (Trastuzumab), an anti-p185ErbB2 murine monoclonal antibody, resulted in decreased levels of β -catenin, Akt and phosphorylated Akt as compared with a single treatment with Ad.*mda-7* or Herceptin (112). These studies suggested that Ad.*mda-7* plus Herceptin might be more efficacious for the therapy of Her-2/neu-overexpressing breast cancer than a single treatment modality. Combination treatment of NSCLC cells with Ad.*mda-7* and gefitinib can reverse resistance to either agent alone. Gupta et al. showed that treatment with recombinant MDA-7/IL-24 proteins [either GST-MDA-7 or GST-M4 (a truncated version of wild type *mda-7/IL-24* exhibiting similar antitumor efficacy) and erlotinib (Tarceva) at sub-optimal apoptosis-inducing concentrations synergistically enhanced growth inhibition and apoptosis over that observed with either agent alone (115). Ad.*mda-7* can also reverse multidrug resistance in colorectal cancer cells and interestingly, drug-resistant cancer cells expressing a multidrug resistance gene (*mdr1*) encoding P-glycoprotein are more susceptible to Ad.*mda-7* than *mdr1* non-expressing drug-sensitive cells indicating a potential benefit of administering Ad.*mda-7* to cancer patients with recurrent drug resistant tumors mediated by overexpression of P-glycoprotein (*mdr-1*) (46). In a recent study conducted by Zheng et al., MDA-7/IL-24 demonstrated a significant effect in overcoming temozolomide resistance in human melanoma cell lines (116).

3: MDA-7/IL-24 and ‘Bystander’ antitumor activity

MDA-7/IL-24 binds to currently recognized MDA-7/IL-24 receptor complexes consisting of two sets of heterodimeric chains, IL-20R1/IL-20R2 or IL-22R1/IL-20R2 (23, 78, 117). Most human tissues express the IL-20R1/IL-20R2 complex. IL-22R is found in a few tissues lacking IL-20R2, such as adult and fetal liver, colon, small intestine, and pancreas. A functional set of cell surface receptors can also be found in the majority of human tumor cells (16). Upon ligand binding, both receptors induce activation of STAT3 (23). However,

our previous studies demonstrated that activation of the JAK/STAT pathway is dispensable for Ad.*mda-7*-induced apoptosis, and cell death triggered by intracellular MDA-7/IL-24 protein occurs through a receptor-independent mechanism (16). Initially the ‘bystander’ activity of MDA-7/IL-24 was studied in melanoma cells where the glycosylated MDA-7/IL-24 showed cell death in a dose-dependent manner, which was mediated through the IL-24 receptors (23). Activation of the IL-24 receptors resulted in phosphorylation of STAT3 followed by its translocation into the nucleus where it upregulated Bax and induced apoptosis in melanoma cells. STAT3-mediated cell death induced by MDA-7/IL-24 was different than the other members of IL-10 family where IL-10, -19, -20 and -22 activate STAT3, but this interaction does not induce cell death. Similarly, in normal cells STAT3 is activated by glycosylated MDA-7/IL-24 without inducing cell death. Similarly, a tumor-selective cytotoxic ‘bystander’ role for secreted MDA-7/IL-24 protein was identified through a novel receptor-mediated death pathway in breast cancer cells, wherein the related cytokines IL-24 and IL-10 exhibit antagonistic activity. Su et al. (78) provided evidence of a ‘bystander’ antitumor effect of *mda-7*/IL-24 resulting from secretion of this cytokine by normal cells. Normal cells were infected with Ad.*mda-7* and directly co-cultured with cancer cells or co-cultured with cancer cells that were separated by an agar overlay, providing a diffusion model for testing for cytokine efficacy. Cell viability and apoptosis (direct co-cultivation), anchorage independent growth (agar diffusion), radiosensitivity (agar diffusion) and invasion (direct co-cultivation) of cancer cells *in vitro* were evaluated using appropriate assays. Moreover, the combination of secreted MDA-7/IL-24 and radiation provoked a “bystander” antitumor effect not only in cancer cells that were inherently sensitive to either *mda-7*/IL-24 or radiation as a single agent, but also in prostate tumor cells overexpressing the anti-apoptotic proteins Bcl-2 or Bcl-X_L and displaying resistance to either treatment used alone (66). This study highlights the significance of normal cells as a resource for producing *mda-7*/IL-24 that can facilitate the eradication of local and systemic tumors (22).

Sauane et al. (67) recently documented that recombinant MDA-7/IL-24 protein can robustly induce expression of endogenous *mda-7*/IL-24, which generates the signaling events necessary for “bystander” killing (Figure 2). This study confirmed that exogenous MDA-7/IL-24-mediated up regulation of *mda-7*/IL-24 was essential for recombinant MDA-7/IL-24-induced apoptotic effects. Blocking *mda-7*/IL-24 by siRNA inhibited extracellular MDA-7/IL-24 receptor-mediated apoptosis. To evaluate the mechanisms underlying this positive autocrine feedback loop this study showed that MDA-7/IL-24 protein induced its own mRNA stabilization without activating the promoter and this posttranslational modification depended on *de novo* protein synthesis. As a consequence of this positive autocrine feedback loop, the secreted MDA-7/IL-24 up regulated or activated its target proteins BiP/GRP78, GADD153, GRP94 and phospho-eIF2 α by inducing an ER stress response as well as the generation of ROS. These results indicate that MDA-7/IL-24 protein induces “bystander” antitumor effects through an ER stress mechanism mediated by a robust activation of its own protein expression, and provides a unified model of *mda-7*/IL-24 action evoking apoptosis selectively in cancer cells.

4: *mda-7*/IL-24 an effective gene therapy for diverse cancers

4.1.1: Cancer Terminator Virus (CTV): cancer-selective virus replication for enhanced delivery of *mda-7*/IL-24

The poor response of many aggressive cancers to radiotherapy and chemotherapy mandates development of targeted, nontoxic and more efficacious treatment protocols. Conditionally replication competent adenoviruses (CRAds) that induce oncolysis by cancer-specific replication are currently being evaluated in clinical trials. However, a single modality approach may not be sufficient to completely eradicate cancer in a patient, because most

cancers arise from defects in multiple genetic and signal transduction pathways. The promoter region of rodent progression elevated gene-3 (PEG-3), cloned and characterized in our laboratory, embodies the unique property of selective activity in a broad-range of tumor cells, both rodent and human, when compared to normal cellular counterparts (118). Bipartite adenoviruses were engineered to express the E1A gene, necessary for viral replication, under control of the PEG-3 promoter (PEG-Prom) and simultaneously express *mda-7/IL-24* in the E3 region (Ad.PEG-E1A-*mda-7*), which was termed the Cancer Terminator Virus (CTV) (43). The efficacy of this unique CTV was evaluated in breast (43) and prostate cancers (72) and also in melanomas (28). Infection of this CTV (designated Ad.PEG-E1A-*mda-7*) in normal mammary epithelial cells and breast cancer cells confirmed cancer-cell-selective adenoviral replication, *mda-7/IL-24* expression, growth inhibition and apoptosis induction. Injecting Ad.PEG-E1A-*mda-7* into human breast cancer xenografts established on both sides of athymic nude mice completely eradicated not only the primary injected tumor on one flank, but also distant tumors (established on the opposite flank of the animal) thereby implementing a 'cure'. Furthermore, the CTV also completely eradicated therapy-resistant Bcl-2 and Bcl-X_L over expressing prostate cancer cells both *in vitro* and *in vivo* (72). Similarly, the ZD55 vector, which contains a deletion of the adenoviral E1B 55-kDa gene, to regulate replication in cancer cells with p53 dysfunction, has been modified to deliver *mda-7/IL-24* (ZD55-IL-24) (119). In spite of leakiness of MDA-7/IL-24 in normal cell, no apparent toxicity was detected in normal lung fibroblast cells, which supports the cancer-specific activity of this novel cytokine. ZD55-IL-24, efficiently killed human colorectal cancer cells by activation of caspases 3 and 9, induction of bax and apoptosis. Moreover, infection of established SW620 colorectal tumors with ZD55-IL-24 showed a much stronger antitumor effect than observed with Ad.IL-24 (a non-replicating virus expressing *mda-7/IL-24* similar to Ad.*mda-7*) or ONYX-015 (a virus preferentially replicating in cells with defective p53). These provocative studies indicate that administering *mda-7/IL-24* using conditionally replicating cancer-specific adenoviruses, the CTV, represents a potentially viable strategy for increasing the therapeutic efficacy of this novel cytokine.

4.1.2: Targeting the tumor and its microenvironment for delivery of *mda-7/IL-24* using a microbubble plus ultrasound approach

A major challenge for effective gene therapy using conditionally replicating Ads is the ability to specifically deliver nucleic acids directly into diseased tissue. Progress in gene therapy has been hampered by concerns over the safety and practicality of viral vectors, particularly for intravenous delivery, and the inefficiency of currently available non-viral transfection techniques (120). Recombinant Ads are one of the most common gene transfer vectors utilized in human clinical trials, but systemic administration of this virus is limited by host innate and adaptive antiviral immune responses, which can limit and/or preclude repetitive treatment regimens (121). Ultrasound (US) contrast agents and US-targeted microbubble destruction (UTMD) represents an innovative and viable method for systemic gene delivery (120–122). Greco et al. (123) demonstrated that microbubble/Ad.*mda-7* complexes targeted to DU-145 cells using US dramatically reduce tumor burden in xenografted nude mice. Intriguingly, US guided microbubble/CTV delivery completely eradicated not only treated DU-145/Bcl-X_L-therapy resistant tumors, but also untreated distant tumors (established in the opposite flank), thereby implementing a 'cure'. These findings advocate potential therapeutic applications of this novel image-guided gene therapy technology for advanced prostate cancer patients with metastatic disease.

4.1.3: Enhanced therapeutic delivery of *mda-7/IL-24* by a tropism-modified adenovirus

Successful gene therapy predominantly relies on efficient and enhanced transgene delivery approaches. Serotype 5 adenovirus (Ad.5) is routinely used as a vector for transgene

delivery. However, the infectivity of Ad.5 is dependent on Coxsackie-adenovirus receptors (CARs); many tumor types show a reduction in this receptor *in vivo*, thereby limiting therapeutic gene transduction. Serotype chimerism is one approach to circumvent CAR deficiency; this strategy is used to generate an Ad.5/3-recombinant Ad that infects cancer cells through Ad.3 receptors in a CAR-independent manner (74). In low CAR human prostate cancer cells (PC-3) (124), a recombinant Ad.5/3 virus delivering *mda-7/IL-24* (Ad.5/3-*mda-7*) is more efficacious than an Ad.5 virus encoding *mda-7/IL-24* (Ad.5-*mda-7*) in infecting tumor cells, expressing MDA-7/IL-24 protein, inducing cancer-specific apoptosis, inhibiting *in vivo* tumor growth and exerting an antitumor 'bystander' effect in a nude mouse xenograft model (74). Similarly, due to insufficient adenovirus serotype 5 gene delivery therapeutic approach has shown limited success in GBM, but Ad.5/3-*mda-7* more effectively infected and killed GBM cells *in vitro* and *in vivo* than Ad.5-*mda-7* (107).

5: Overcoming an exception to the ubiquitous antitumor properties of *mda-7/IL-24*: a chemoprevention gene therapy (CGT) approach for pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is a very aggressive and complex cancer in which numerous subsets of genes undergo genetic change, either activation or inactivation, in a temporal manner during tumor development and progression. Common genetic modifications in PDAC include early activation of the *K-ras* oncogene (85 to 95%), overexpression of specific growth factors and their associated receptors and inactivation of the p16/RB1 (>90%), p53 (75%), DPC4 (55%) and *BRCA2* tumor suppressor genes (125). Despite the provocative antitumor effects of *mda-7/IL-24* in multiple cancer subtypes, Ad.*mda-7* infection of PDAC cells remains largely ineffective in promoting growth inhibition and fails to induce cell death (99, 126). We observed that activated *K-ras* can cause a preferential 'translational block' of *mda-7/IL-24* mRNA and inhibition of *K-ras* allows *mda-7/IL-24* protein translation with resultant apoptosis (126). Accordingly, we have demonstrated previously that a combinatorial treatment (Figure 3) employing *mda-7/IL-24* and antisense inhibition of *K-ras* or inhibition of *K-ras*-downstream extracellular regulated kinase 1/2 (ERK1/2) signaling induces apoptosis in *K-ras* mutant pancreatic carcinoma cells and inhibits growth of these tumor cells in xenograft murine models (127). Based on these intriguing observations we have endeavored to improve this combinatorial approach by incorporating both genetic constructs in the same delivery vehicle and have engineered a bipartite adenovirus, Ad.m7/KAS that can simultaneously express *mda-7/IL-24* and extinguish *K-ras* expression (99). PDAC cells containing a mutant *K-ras* (including PANC-1, MIA PaCa-2, and AsPC-1) are exquisitely sensitive to Ad.m7/KAS resulting in profound growth suppression and induction of apoptosis both *in vitro* and *in vivo* in human PDAC xenografts in nude mice (99). In contrast, PDAC cells containing a wild-type *K-ras* gene also display a 'translational block' of *mda-7/IL-24* mRNA that is not reversed by inhibiting *K-ras*, indicating that additional molecular alterations contribute to this inhibitory process in wild-type PDAC cells.

Of potential clinical relevance, a dietary monoterpene, perillyl alcohol (POH), even at low doses significantly augmented gene therapy by making inherently resistant pancreatic carcinoma cells sensitive to Ad.*mda-7* therapy. Subsequent detailed mechanistic studies of this chemoprevention gene therapy (CGT) approach indicated that the generation of reactive oxygen species mediated by xanthine oxidase, a major source of superoxide radical production, by POH in combination with Ad.*mda-7* was a significant contributory factor in the cancer-specific toxicity (128, 129). Additionally combinatorial treatment of pancreatic carcinoma cells with POH and Ad.*mda-7* resulted in the association of *mda-7/IL-24* mRNA with polysomes and concomitant translation of this normally inefficiently translated

message in pancreatic cancer cells into functional protein (Figure 3). This study supported the CGT approach as a novel strategy of combining a dietary agent with gene therapy as a potent inhibitor of pancreatic cancer cell growth and survival. Since both POH and Ad.*mda-7* are in the clinic without promoting overt toxicity in cancer patients, we anticipate that evaluation of this novel strategy will facilitate development of an efficient, selective and non-toxic approach for initiating a Phase I/II clinical trial to effectively treat PDAC patients.

6: The clinical experience with *mda-7/IL-24* in a Phase I clinical trial of patients with advanced cancers

Based on its ubiquitous anti-tumor activity in multiple cancer indications *in vitro* cancer cell culture systems, which translated into profound anti-cancer activity in nude mouse human tumor xenograft models, *mda-7/IL-24* has now taken the noteworthy and mandatory step for a putative cancer gene therapeutic by entering the clinic (80–83, 130, 131). These studies indicated that when *mda-7/IL-24* was repeatedly administered intratumorally using a replication incompetent adenovirus it was safe and displayed significant clinical efficacy (80, 81, 130, 131). The Phase I clinical trial recapitulated many of the observations initially uncovered using cell culture and animal tumor models, i.e., effective induction of tumor cell-specific apoptosis, potent antitumor ‘bystander’ effect, and immune modulation (7, 83, 84). In this trial, 28 patients with resectable solid tumors received intratumoral injections with Ad.*mda-7* (INGN 241) (81, 82). In 100% of injected lesions, vector transduction, transgene mRNA, elevated MDA-7/IL-24 protein, and apoptosis induction were evident. In specific instances, a single injection with Ad.*mda-7* (INGN 241) resulted in transduction of 10% to 30% of the tumor mass with 70% of the tumor cells displaying signs of apoptosis, supporting the antitumor ‘bystander’ effect observed originally in cell culture models (67, 78) and in *in vivo* animal models (28, 43, 72). Multiple injections with Ad.*mda-7* (INGN 241) were found to be safe with toxicity being self-limiting and generally mild. Moreover, from this study it is now evident that *mda-7/IL-24* was well tolerated, capable of inducing apoptosis in a significant portion of tumor cells, and this therapy displayed evidence of clinically significant activity. Additionally, this phase I clinical trial (82, 83) also supported the immune-modulating properties of *mda-7/IL-24*, including transient increases in serum levels of IL-6, IL-10, and tumor necrosis factor- α with marked increases in CD3+ CD8+ T cells (suggesting enhanced TH1-like response and activated CD8+ T cells). Taken together, these findings inspire optimism that *mda-7/IL-24* may provide significant benefit for patients with multiple types of cancer, especially in the context of improved tumor delivery and by applying combinatorial approaches.

Summary and Future Perspectives

The unique and multifaceted anti-cancer properties of *mda-7/IL-24* and its robust activity in both animal models and a Phase I clinical trial implies that this gene might provide an excellent gene therapy for cancer. In addition to cancer-specific cytotoxicity, potent ‘bystander’ activity and induction of antitumor immunity, *mda-7/IL-24* also synergizes with radiation and other chemotherapeutic agents further amplifying the potential therapeutic applications of this novel cytokine. To produce an optimum therapeutic response using *mda-7/IL-24*, defining ways of ensuring efficient delivery of the molecule to the target cancer tissue would be beneficial. Additionally, combining *mda-7/IL-24* with agents targeting specific signaling pathways that are defective in cancer cells could augment the induction of ER stress in tumor cells thereby further enhancing the anticancer activity of this therapeutic molecule. Current studies are focused on: 1) increasing Ad entry into cancer cells in a CAR-independent manner, which would diminish an important obstacle to effective *in vivo* delivery of therapeutic viruses expressing *mda-7/IL-24*; 2) developing approaches for improving systemic administration of Ads and avoiding other problems

preventing optimum utilization of Ads or CRAds for systemic therapy with *mda-7/IL-24*, such as squelching of Ad by the liver, immune system-mediated clearance of circulating Ad and developing anti-Ad antibodies precluding multiple administrations; 3) inventing novel systemic approaches, such as microbubble/ultrasound delivery, to administer *mda-7/IL-24*, as a virus or purified protein, specifically to tumor cells or its' microenvironment; 4) identifying small molecules using high throughput screening approaches which can stimulate *mda-7/IL-24* expression specifically in target cells or their *milieu*, which could either be cancer cells or normal cells in the cancer microenvironment; 5) exploring new ways of delivering *mda-7/IL-24* to tumors or their *milieu* using stem cells and dendritic cells; 6) using genetic engineering and rational structural design to create new molecules that will behave in a manner similar to *mda-7/IL-24* with the added benefit of displaying increased stability in serum and enhanced efficacy against cancers; and 7) using chemoprevention plus virus gene delivery as a novel approach to both prevent and treat pancreatic cancer by promoting ROS production in PDAC. Based on the early success of *mda-7/IL-24* as a potentially effective anticancer therapy in patients, we are optimistic that with appropriate modifications this cytokine may become a frontline therapeutic for multiple human cancers.

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Biographies

Rupesh Dash received his PhD in Biotechnology from the Indian Institute of Technology, Kharagpur, India. Since 2008, he has been a post Doctoral Research Scientist in the Department of Human and Molecular Genetics in Virginia Commonwealth University, School of Medicine, Richmond, VA. Overall, his research focuses on developing therapeutics for prostate carcinomas by targeting pro-survival members of the Bcl-2 family

particularly myeloid cell leukemia-1 (Mcl-1), which enhance the biological activity of the tumor suppressor gene melanoma differentiation associated gene-7/Interleukin-24 (*mda-7/IL-24*). The primary research objectives are to design viable and effective gene therapy options, which can be translated into the clinic for prevention and therapy of cancer using this novel and unique IL-10 gene family cytokine, *mda-7/IL-24*.

Paul B. Fisher, MPh, PhD, is Professor and Chair of the Department of Human and Molecular Genetics at VCU School of Medicine, Director of the VCU Institute of Molecular Medicine (VIMM) and the Thelma Newmeyer Cowmen Chair in Cancer Research in the Massey Cancer Center. Dr. Fisher's laboratory studies the genetic/molecular/biochemical basis of cancer development and progression to metastasis and is designing improved methods for cancer detection, chemoprevention and therapy. His laboratory pioneered subtraction hybridization as an approach to identify and clone novel genes involved in important physiological processes. Using this approach his research group was the first to clone: the cyclin-dependent kinase inhibitor p21 as melanoma differentiation-associated gene-6 (*mda-6*); the novel IL-10 gene family member *mda-7/IL-24* that selectively induces apoptosis or toxic autophagy uniquely in multiple cancers without affecting normal cells; *mda-5*, a patent receptor for double-stranded RNA that is a key component of the innate immune process; human polynucleotide phosphorylase, a RNA degrading enzyme that targets specific RNAs such as c-myc and specific mature miRNAs for destruction and induces cellular senescence; astrocyte elevated gene-1 (AEG-1) a unique gene that is upregulated in multiple cancers (including those of brain, breast, esophagus, prostate and liver) and is a potential therapeutic target; and genes upregulated or downregulated during cancer progression (progression elevated genes, PEG, and progression suppressed genes, PSG, respectively). He has over 30 years of research experience and is among the top 5% of NIH funded investigators over the past 25 years. Dr. Fisher has published over 400 primary papers and reviews, served on numerous NIH study sections and government and private grant review panels, over 50 issued patents and was the founder of GenQuest Inc. a functional genomics company that merged with Corixa Corporation which traded on NASDAQ and was recently acquired by Glaxo-SmithKline.

Glossary

VEGF	Vascular endothelial growth factor
TGFβ	Transforming growth factor beta
PI3K	Phosphatidylinositol 3-kinase
MMP-2	matrix metalloproteinase-2
JNK	c-Jun N-terminal kinase
PP2A	Protein phosphatase 2
BiP/GRP-78	Binding immunoglobulin protein/ 78 kDa glucose-regulated protein
GRP94	94 kDa glucose-regulated protein
PKR	Protein kinase R

PERK	protein kinase-like endoplasmic reticulum kinase
ATG-5	Autophagy protein 5
Bcl-2	B-cell lymphoma 2
CD95	Fas receptor
FasL	Fas ligand
FADD	Fas-Associated protein with Death Domain
ATF	Activating transcription factor
Bax	Bcl-2-associated X protein
Bak	Bcl-2 homologous antagonist/killer
Mcl-1	myeloid cell leukemia-1
p-38 MAPK	P38 mitogen-activated protein kinase
eIF2	Eukaryotic Initiation Factor 2
TyK2	Non-receptor tyrosine-protein kinase
GADD153/34/45	Growth arrest and DNA-damage-inducible genes

Figure 1. Model of *mda-7/IL-24*-induced apoptosis in cancer cells

Outline of proposed selective cytotoxic effects of *mda-7/IL-24* on cancer versus normal cells. When ectopically over expressed, MDA-7/IL-24 localizes in the ER/Golgi compartments, regardless of the presence or absence of a secretory signal in the protein. Accumulation of MDA-7/IL-24 protein in transformed/tumor cells in this compartment triggers apoptosis, autophagy and induction of ceramide that could involve induction of ER stress and/or reactive oxygen species in mitochondria. MDA-7/IL-24 activates signal transduction pathways and/or potentially enters cancer cells and activates pro-apoptotic pathways by localization and accumulation in the ER/Golgi compartment and/or by inducing mitochondrial dysfunction. A combination of pathways triggered by *mda-7/IL-24* results in transformed cell-specific apoptosis. Modified from Lebedeva *et al* (7).

Figure 2. Model illustrating the possible molecular mechanism of cancer cell-selective apoptosis induction by recombinant MDA-7/IL-24 protein

Recombinant MDA-7/IL-24 protein triggers ER stress and ROS production. (A) Cells were treated with recombinant MDA-7/IL-24 protein (20 ng/ml) and Western-blotting analyses evaluated levels of BiP/GRP78, GRP94 and phospho-eIF2α proteins. (B) Cells were untreated or treated with MDA-7/IL-24 protein (20 ng/ml) for 24 and 48 h, and ROS production was determined as described in (67). Results are average from at least three independent experiments. (C) Model illustrating the possible molecular mechanism of cancer cell-selective apoptosis induction by recombinant MDA-7/IL-24 protein. Reproduced, by permission of the publisher, from Sauane et al. (67).

Figure 3. Proposed model of K-ras AS or POH plus Ad.mda-7 apoptosis-inducing activity in pancreatic carcinoma cells

Infection of Ad.mda-7 in pancreatic carcinoma cells results in *mda-7/IL-24* mRNA, but no protein or inhibition of growth or induction of apoptosis. Ad.m7/KAS (a bipartite adenovirus that expresses both *mda-7/IL-24* and K-ras AS) or POH treatment of Ad.mda-7-infected pancreatic carcinoma cells results in *mda-7/IL-24* mRNA association with polysomes and production of MDA-7/IL-24 protein. This combination treatment results in induction of ROS, which mediates apoptosis. Reproduced, by permission of the publisher, from Lebedeva et al. (127).

Table 1

Ad.mda-7 selectively inhibits growth and induces apoptosis in a wide spectrum of human cancer cells without affecting normal cells

Normal Cells	Cancer Cells
Mammary Epithelial Cells (HuMEC)	Breast Carcinoma
Prostate Epithelial Cells (HuPEC)	Prostate Carcinoma
Melanocytes (NHuMel)	Melanoma
Bronchial Epithelial Cells (NHBE)	Lung Cancer
Fetal Astrocytes (PHFA)	Glioblastoma Multiforme
Skin Fibroblasts (MJ90)	Osteosarcoma
Skin Fibroblasts (HF)	Colon Carcinoma
Lung Fibroblasts (NHLF)	Nasopharyngeal Carcinoma
Endothelial cells (HuVEC)	Cervical Carcinoma
Ovarian Epithelial Cells	Ovarian Carcinoma
Renal Epithelial cells	Renal Carcinoma*
Mesothelial cells	Gastric Carcinoma
Hepatocytes	Hepatocellular Carcinoma
Pancreatic Mesenchymal Cells	Pancreatic Carcinoma [#]

* GST-MDA-7 has been evaluated.

[#] In pancreatic carcinoma cells containing a mutant *K-ras* gene, the combination of *Ad.mda-7* and inhibition of mutant *K-ras* or treatment of POH induces growth inhibition. *Ad.mda-7* alone or in combination with inhibition of mutant *K-ras* fails to induce growth inhibition or apoptosis in BxPC-3 pancreatic carcinoma cells, which contain a wild-type *K-ras* gene, whereas the combination of *Ad.mda-7* and POH selectively kills all pancreatic cancer cells irrespective of their *K-ras* status.