Proceedings of the National Conference
On Undergraduate Research (NCUR) 2014
University of Kentucky, Lexington, KY
April 3-5, 2014

An Immunotherapy Against Cancer Using Viral RNA

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Abstract

In the human immune system, receptors and phosphokinase signaling cascades alert the body of the presence of a virus infection. Recognized by cellular pattern-recognition receptors (PRR), viral RNA species trigger the activation of type I interferon (IFN-beta) and type III (IFN-lambda) innate immune pathways. IFN induction ultimately leads to expression of antiviral genes that inhibit virus replication, and to adaptive cytotoxic T lymphocyte (CTL) responses that eliminate infected cells. We hypothesize that an antiviral response triggered by viral RNA could directly activate an oncolytic pathway that kills tumor cells, or exposes tumor cells to antiviral CTL that are capable of targeting cells expressing tumor antigens on MHC I complexes on the cell surface. Synthetic viral RNA (poly-I:C) was transfected into a panel of human lung and pancreatic tumor cell lines. Transfected cells were assayed to determine interferon response by quantitative RT-PCR. In human lung adenocarcinoma (A549) cells, poly I:C containing both low-molecular weight (LMW) and high-molecular weight (HMW) chains induced 1.6-4 fold expression of IFN-beta, IFN-lambdas (IL28 and IL29), and the antiviral gene MXA1, suggesting that this tumor cell type contains an intact PRR signaling pathway. LMW poly-I:C induced 1-3 fold expression, while HMW poly-I:C showed 1.5-2.5 induction. Assays to measure cell death and growth inhibition in A549, as well as IFN responses in other human lung (NCI-H23) and pancreatic (PaCa-2) tumor cells are ongoing. By understanding IFN responses in human tumor cells, this project harnesses the antiviral capabilities of the immune system to develop new immunotherapies against cancer.

Keywords: Immunotherapy, Cancer, Virolytic oncotherapy

1. Introduction

Innate and adaptive immune responses are the body's defenses against invading pathogens, such as viruses and bacteria. Early recognition of non-self pathogen-associated molecular patterns (PAMPs) is a key facet of innate immunity, particularly against RNA virus infections. Cellular pattern-recognition receptors (PRR) such as Rig-I and TLR3 recognize virus RNA PAMPs and signal to downstream proteins, leading to the induction of type 1 interferon (IFNß/α). IFN α signals to the infected cell and to neighboring cells in a paracrine manner, activating hundreds of antiviral genes and signaling to innate and adaptive immune cells. Antigen-presenting cells such as dendritic cells (DC) present virus protein epitopes for the development of adaptive cytotoxic T-cell (CTL) and antibody responses. CTL specifically target and induce cell death of infected cells presenting virus epitopes on MHCI. Interestingly, experimental RNA virus infection of malignant tumor cells in mouse models leads to a CTL response that eliminates the tumor[4]. However, in humans, malignant tumors appear to have escaped anti-tumor CTL immune surveillance, likely because non-self PAMPs or epitopes are not present.

The goal of this project is to activate type I, type III, and Interferon Stimulated Genes (ISG) in tumor cells, in order to trigger the immune system to target those tumor cells in a manner similar to how it targets virus-infected cells. We have focused on developing a novel immunotherapy strategy using viral RNA to activate the Interferon

pathways in tumor cells, to thus help elicit anti-viral innate immune and CTL responses against the tumor cells. Type I and Type III pathways were activated using purified viral RNA and synthetic RNA such as poly-I:C, in a panel of human tumor cell lines including lung carcinomas and pancreatic cancer.

Methods were tried empirically in transformed human cell lines such as 293T, which showed successful IFN-beta induction. IFN responses and cytotoxicity was measured by RT-PCR, CellTiterGlo, and Caspase 3/7, as well as a luciferase reporter system using appropriate controls. Other innate immune responses, such as NFkB inflammatory signaling, MHCI antigen presentation, and inflammasome responses, stem off this study and are involved in future studies. Viral RNA and tumor types exhibiting significant IFNß/α induction will be tested in future experiments for recognition by mouse CTL, and results analyzed for possible immunotherapy strategies with primary human tumor cell explants.

Toll receptors on the surface of cells, as well as cytoplasmic receptors such as RigI, are able to detect the presence of viral RNA, due to the three phosphates on the 5' end of the viral RNA[3]. Viral RNA containing a 5' triphosphate are differentiated from most cellular RNA that only has a single phosphate on the 5' end (tRNA, ribosomal RNA), or are capped (mRNA). The detection of viral RNA by these receptors triggers signal cascades that activate type 1 interferons, leading to amplification of the signal, paracrine signals sent to neighboring cells, and an innate immune response. Type I interferon activation and amplification leads to activation of interferon stimulated genes (ISG), which are antiviral genes. An "antiviral state" is established in cells expressing ISG, such as RNAseL and PKR[14]. With activation of the antiviral genes, the adaptive immune system is activated, with engagement of macrophages and dendritic cells leading to cytotoxic T cell (CTL) and antibody responses. The immune system thus declares war on the virus-infected cells, and prevents further replication or translation of viral RNA, or kills the cell. In tumor cells, this pathway could be activated, with the interferon activation leading to an immune response to kill the tumor cells infected with viruses.

Pancreatic cancer specifically is a cancer with a poor prognosis, as it metastasizes quickly and aggressively. In 2008, pancreatic cancer was the fourth leading cause of deaths from cancer[13]. Treatment for pancreatic cancer includes chemotherapy and radiation, as well as surgery if the tumor is resectable, using techniques such as the Whipple procedure, or a pancreatectomy[13]. Even when treated surgically, the outcome is not normally favorable. Oncolytic virotherapy may prove to be a successful treatment method for this cancer.

The first specific aim was to induce induction of type I and type III interferon response in human tumor cells transfected with viral RNA. This was tested by transfecting multiple cell lines with Sendai Virus (SeV) RNA and poly I:C, synthetic viral RNA. Interferon induction of both type I and type III was measured by RT-PCR.

The second specific aim was to measure induction of cell death pathways, cell growth inhibition, and MHC1 expression. In vitro tumor growth assays were used to analyze cell viability and growth of transfected tumor cells, using CellTiterGlo for ATP availability and Caspase 3/7 for apoptosis induction.

2. Methods

2.1 Cell Culture:

Cells were cultured according to ATCC protocol. A549 lung adenocarcinoma cells were grown in DMEM media with 10% fetal bovine serum (FBS) and antibiotics. Passaging followed ATCC guidelines and transfection was accomplished using 10% FBS DMEM without antibiotics. PaCa-2 pancreatic carcinoma cells were cultured with 10% FBS DMEM. Other cells used followed ATCC protocols. NCI-H23 lung adenocarcinoma cells, Calu-3 lung carcinoma cells, and 293T human kidney cells were also cultured.

2.2 Transfection:

Cells were transfected with SeV, poly I:C low molecular weight (LMW) and high molecular weight (HMW). Transfected cells were harvested and RNA was extracted for PCR and for Caspase 3/7 and CellTiterGlo assays.

2.3 RT-PCR:

Reverse transcriptase polymerase chain reaction was used to determine induction of type I and type III interferons, as well as induction of MXA1, an interferon stimulated gene.

2.4 Assays:

CellTiterGlo determined ATP availability. Caspase 3/7 measured levels of apoptosis induction in transfected cells. Luminescence was measured using the BioTek plate reader.

3. Results

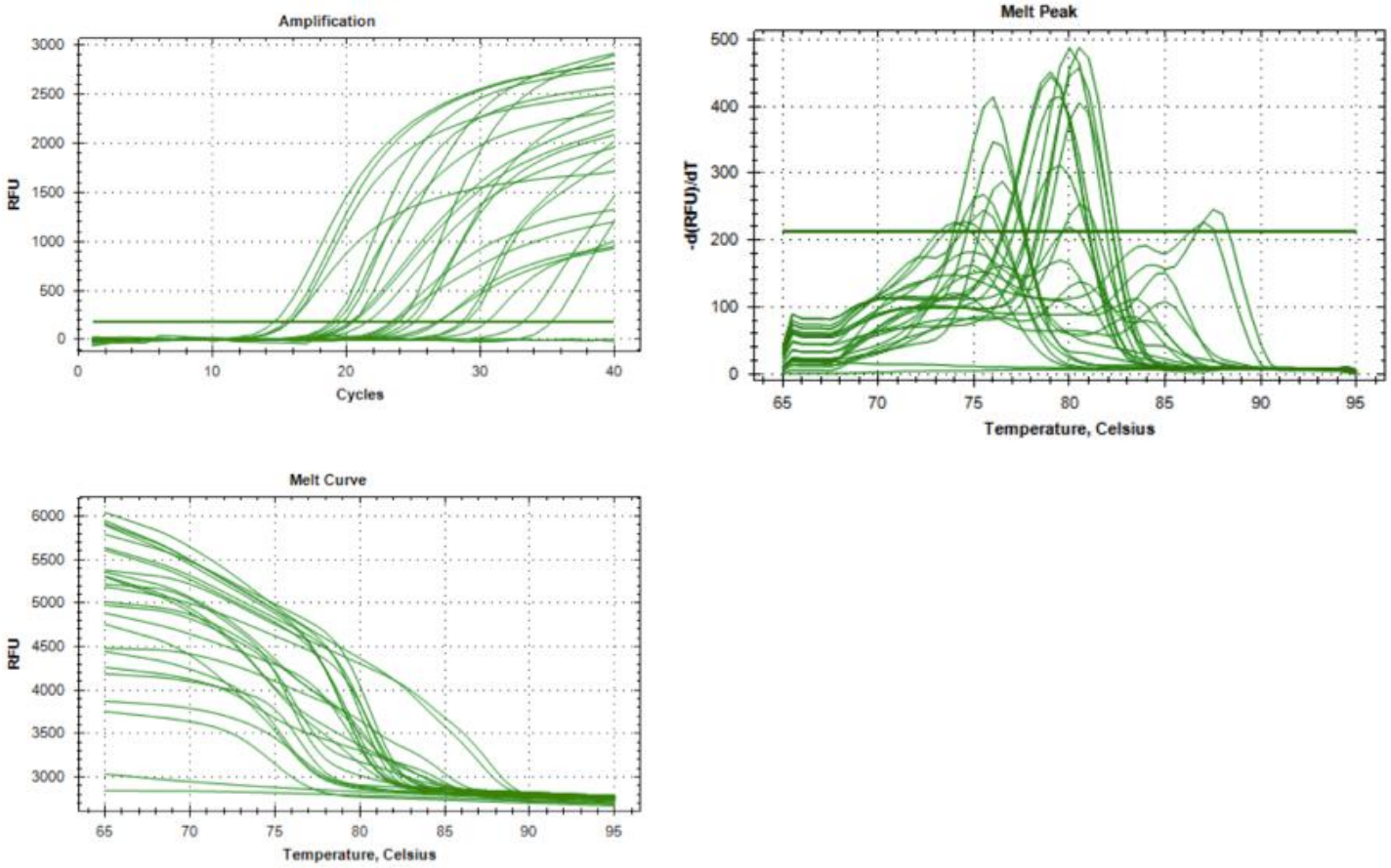

Figure 1: RT-PCR results of PaCa-2 transfection.

Amplification curve shows good curves as does the melt curve. Melt peak shows majority of the peaks above the line. Delta delta CT analysis was done on the data in order to more visually compare results, showing induction of type I and type III interferons when using LMW, and weak induction of MXA1. There was also weak induction of HMW type I and type III interferon response.

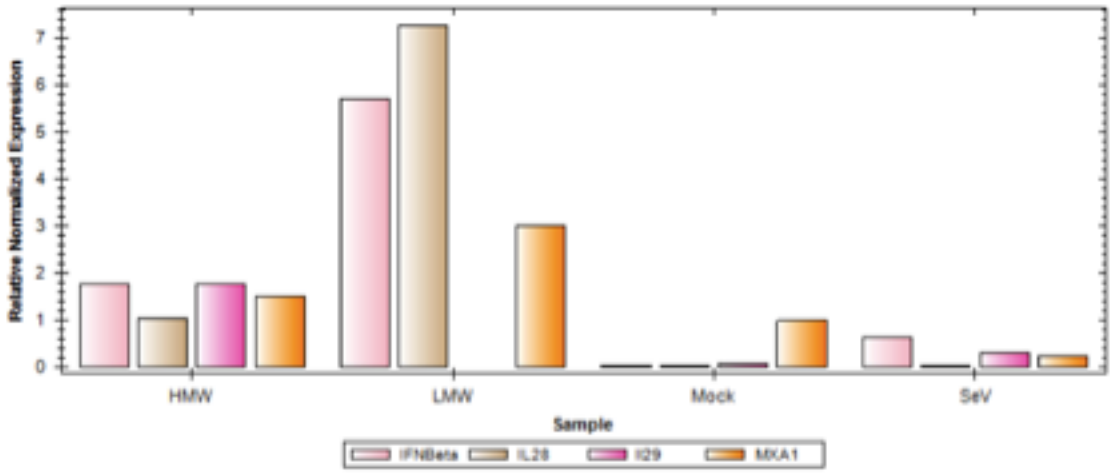

Figure 2: Delta delta CT analysis of PaCa-2 transfection with Poly I:C HMW, LMW, and SeV.

Results show that LMW induced type I and type III interferons strongly with weak induction of MXA1 compared to mock. HMW had some weak induction but PaCa-2 are most responsive to LMW.

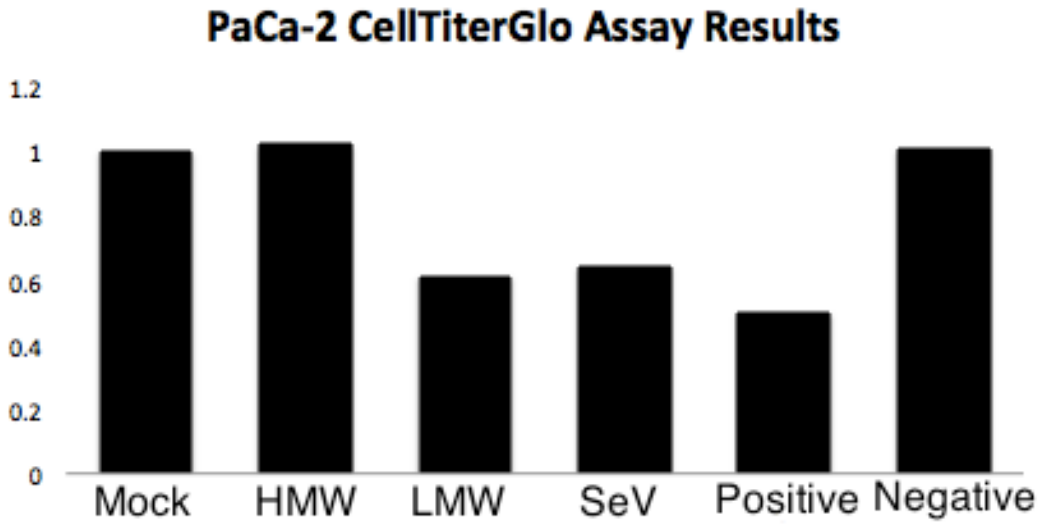

Figure 3: HMW and SeV induce apoptosis in PaCa-2 transfected cells.

Positive control with Doxorubicin induced apoptosis in this type of cell about the same amount as the Mock. HMW and SeV, however, induced apoptosis, while LMW did not induce apoptosis at all. In these results the synthetic viral RNA had a greater degree of induction than the chemotherapy agent, Doxorubicin.

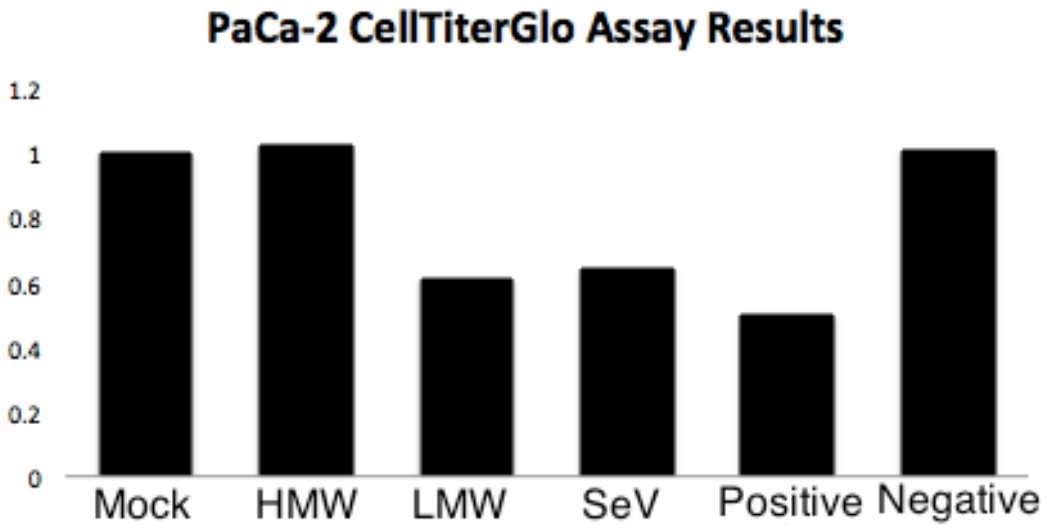

Figure 4: LMW and SeV decrease ATP availability in PaCa-2 transfected cells.

While HMW and SeV both induce apoptosis (Figure 3), HMW did not reduce ATP availability. SeV and LMW both reduce ATP availability and thereby increase cell growth inhibition, although to a slightly lesser extent than with the positive control with Doxorubicin. LMW, through PCR, was shown to induce type I and type III interferons, and it does reduce cell viability, but likely through a different path than apoptosis.

4. Discussion

PaCa-2 (pancreatic) cells were transfected with Poly I:C HMW, LMW, and a non-replicating form of Sendai virus. Q-PCR was performed and melt curve and peak were good as in Figure 1, indicating that the Poly I:C LMW induces type I and type III interferons. There appeared to be weak induction of MXA1 as well, and HMW seemed to weakly induce type I and type III interferons.

Delta delta CT analysis was performed and was graphed as shown above in Figure 2. When compared to mock, this clearly showed induction of type I and type III interferon in the cells which had been transfected with Poly I:C LMW. There was also weak induction of the MXA1 gene. HMW did weakly induce type I and type III interferons, while Sendai virus did not induce anything when compared to the mock.

A Caspase 3/7 assay was performed as in Figure 3, using a positive control with Doxorubicin, a chemotherapy drug. Caspase 3/7 assays measure degree of apoptosis in cells. Both HMW and Sendai virus induce apoptosis as indicated on the graph, and to a greater degree than the Doxorubicin. LMW did not induce apoptosis at all. In this case, these PaCa-2 cells transfected with synthetic viral RNA showed a greater degree of induction of apoptosis than did the Doxorubicin.

Next, as shown in Figure 4, a CellTiterGlo assay was performed. This measures ATP availability, and Doxorubicin was again used as a positive control. Sendai virus and LMW were both found to decrease ATP availability, which thereby increases inhibition of cell growth. However, this inhibits cell growth to a lesser degree than the Doxorubicin. Comparatively, HMW does not reduce ATP availability, although as in Figure 3, it does induce apoptosis. Sendai virus can induce apoptosis and decrease ATP availability in this case, while LMW does reduce cell viability but likely through a different avenue than through apoptosis. LMW also most strongly induces type I and type III interferons, the first step to triggering the immune system to attack an infected or transfected cell.

5. Conclusion

The focus of this study is to develop a new oncolytic virotherapy method using viral RNA to induce type I and type III interferon pathways in multiple human tumor cell lines. Thus far, in the PaCa-2 cell line, both type I and type III interferons have been induced strongly when using LMW. MXA1 has been weakly induced as well.

Apoptosis was induced in the experiments, but not to as great a degree as when using Doxorubicin. Currently, in this stage, the method would likely not be an effective replacement for any chemotherapy drug. However, likely this immunotherapy method could be used in conjunction with other forms of treatment, including chemotherapy and radiation.

Tumor cell lines, and potentially tumor cell types, may respond differently to immunostimulatory viral RNA, by nature of their mutagenic nature. Not only are all tumor cell lines different from each other, but also individual tumors vary from person to person. The high mutation rate not only ensures that treatment is difficult to generalize to a large number of people, but also helps the tumor to evade the body's immune system under normal conditions, which is why we are hopeful that using viral RNA to trigger the immune system to recognize these cells as foreign and invaders will be a new form of immunotherapy.

As shown by the Caspase 3/7 and CellTiterGlo assays, not all interferon inducers may be cell death inducers. Tumor cells have developed ways of evading the immune system, and they may be resistant to cell death pathways. Therefore, interferon induction agents may only weakly induce cell death as compared to chemotherapy agents such as Doxorubicin, or may only inhibit cell growth. Not all interferon inducers will induce apoptosis, but they seem to inhibit cell growth.

The poly I:C LMW was able to induce the most significant response in pancreatic cancer (PaCa-2) cells, and reduced cell viability, but was not accomplishing this through a non-apoptosis pathway. This could be a promising immunotherapy method for pancreatic carcinomas, as tumor cells often resist apoptosis.

In conclusion, this project has been able to successfully induce both type I and type III interferons (β and λ respectively) by transfecting human tumor cells with viral RNA. Once the interferons have been induced, which is effectively the first step of the activation of the immune system, apoptosis was ultimately induced in these transfected cells, in some cases to a greater degree than a current chemotherapy drug in use. There were also varying degrees of decrease in ATP availability which leads to cell growth inhibition. Not only does this process of virolytic oncotherapy trigger the immune system to attack its own transfected tumors, but it also leads to complete activation of the immune system, ending with apoptosis or at least cell growth inhibition. Given the varying degrees to which different synthetic viral RNA's used in the project induced different levels of interferon induction or apoptosis, or cell growth inhibition, multiple types of synthetic viral RNA could be used in conjunction to achieve the overall goal of induction of type I and type III interferon followed by apoptosis and/or cell growth inhibition.

Future research includes mapping different induction pathways and responses in different tumor cell lines. There will also be more studies done on MHC1 expression. Eventually, this study hopes to develop an immunotherapy method that may or may not stand on it's own in treatment. Ultimately, this could lead to personalized treatment for a patient's own cancer. Patient biopsies could be sequenced and a synthetic viral RNA could be customized to the patient's specific tumor or cancer. Future work also involves developing a method for ensuring that the synthetic RNA affects only the tumor cells. At present this is a future goal, but some current ideas include customizing receptors.

6. Acknowledgements

This work was funded by the Alaska Heart Institute grant, and the Alaska INBRE grant which funds the lab. I would like to thank the Office of Undergraduate Research and Scholarship and the Honors College at University of Alaska Anchorage who made undergraduate research at UAA possible, with additional thanks to Dean Ronald Spatz. The UAA Discovery grant provided funding to travel to present this research at NCUR 2014 at the University of Kentucky in Lexington. This work would not have been possible without the assistance of Dr. Eric Bortz, Dr. Jonathan Rupp, Nathaniel Ray, Robert Robl, Monica Cox, and Rachel Shercliffe, all of whom worked in the lab as well.

7. Selected References

1. Pitha, P. (2000). Introduction: interferons' connection to cancer. *Seminars in Cancer Biology*, *10*, 69-72. doi: 10.1006/scbi.2000.0309
2. Blankenstein, T., Coulie, P., Gilboa, E., & Jaffee, E. (2012). The determinants of tumour immunogenicity. *Nature Reviews: Cancer*, *12*, 307-313.
3. Baum, A., & Garcia-Sastre, A. (2011). Differential recognition of viral rna by rigI *Virulence*, *2*(2), 166-169.
4. Vigil A., Martinez, O., Chua, M.A., Garcia-Sastre, A. (2008). Recombinant Newcastle disease virus as a vaccine vector for cancer therapy. *Molecular Ther. 16*, 1883-1890.
5. Bernardo AR, JM Cosgaya, A Aranda and AM Jime´nez-Lara. (2013) Synergy between RA and TLR3 promotes type I IFN-dependent apoptosis through upregulation of TRAIL pathway in breast cancer cells. Cell Death and Disease (2013) 4, e479.
6. Li W, Huang X, Liu Z, Wang Y, Zhang H, Tong H, Wu H, Lin S. (2012) Type III interferon induces apoptosis in human lung cancer cells. Oncol Rep. 2012 Sep;28(3):1117-25.
7. Krejcová D, Procházková J, Kubala L, Pacherník J. (2009) Modulation of cell proliferation and differentiation of human lung carcinoma cells by the interferon-alpha. Gen Physiol Biophys. 2009 Sep;28(3):294-301.
8. Dal Col J, Mastorci K, Faè DA, Muraro E, Martorelli D, Inghirami G, Dolcetti R. (2012). Retinoic acid/alpha-interferon combination inhibits growth and promotes apoptosis in mantle cell lymphoma through Akt-dependent modulation of critical targets. Cancer Res. Apr 1;72(7):1825-35.
9. Fujioka N, Ariyasu T, Arai N, Ariyasu H, Yamamoto S, Tanimoto T, Ikegami H, Ikeda M, Ohta T, Fukuda S, Kurimoto M. (2006) Role of p53 in the inhibitory effects of interferon-alpha subtypes on proliferation of hepatocellular carcinoma cells. Biomed Res. Oct;27(5):219-26.
10. Martínez-Gil L, Ayllon J, Ortigoza MB, García-Sastre A, Shaw ML, Palese P. (2012) Identification of small molecules with type I interferon inducing properties by high-throughput screening. PLoS One. 7(11):e49049
11. Bennett RL, Carruthers AL, Hui T, Kerney KR, Liu X, et al. (2012) Increased Expression of the dsRNA-Activated Protein Kinase PKR in Breast Cancer Promotes Sensitivity to Doxorubicin. PLoS ONE 7(9): e46040.
12. Meng, Linxin; Chi, Yuhua; Wang, Xiangxu; Ding, Zhaojun; Mou, Ling; Cui, Wen; Xue, Yingjie. (2012). The neurotorphic factor artemin promotes the motility and invasiveness of MIA PaCa-2 pancreatic cancer cells. Chinese-German Journal of clinical oncology. April 2012, 11: 4. 219-223.
13. Hidalgo, Manuel. (2010). Pancreatic cancer. The New England Journal of Medicine. April 29, 2010; 362; 1605-1617.
14. Bowie AG, Unterholzner L. 2008. Viral evasion and subversion of pattern-recognition receptor signaling. Nat. Rev. Immunol. 8:911-922.