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Survivin: Regulation by YY1 and Role in Pancreatic Cancer Combination Therapy

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Survivin: Regulation by YY1 and Role in Pancreatic Cancer Combination Therapy

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

YY1	Yin-Yang 1
IAP	Inhibitor of Apoptosis Protein
HIF-1α	Hypoxia-Inducible Factor-1 alpha
XIAP	X-linked Inhibitor of Apoptosis
Sp	Specificity protein
Luc	Luciferase
Mut	Mutant
EMSA	Electrophoretic Mobility Shift Assay
DR5	Death Receptor 5
TRAIL	TNF-Related Apoptosis-Inducing Ligand
BCL_{XL}	B-cell Lymphoma- Extra Large
PDC	Pancreatic Duct Cell Carcinoma
IPMT	Intraductal Papillary-Mucinous Tumor
AR	Androgen Receptor
EMT	Epithelial-to-Mesenchymal Transition
ChIP	Chromatin Immunoprecipitation
STAT	Signal Transducers and Activators of Transcription
Bcl-2	B-cell lymphoma- 2
EGF	Epidermal Growth Factor
KLF5	Kruppel-like Transcription Factor 5
Rb	Retinoblastoma
Egr-1	Early Growth Response Protein- 1

ABSTRACT OF THE DISSERTATION

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by

Nicholas R. Galloway

Doctor of Philosophy, Graduate Program in Biochemistry
Loma Linda University, March 2014
Dr. Nathan Wall, Chairperson

Despite significant clinical and basic science advancements, cancer remains a devastating disease that affects people of all ages, races, and background. Survivin, the fourth most common transcript found in cancer cells, is a protein that is thought to be involved in the enhanced proliferation, survival, and metastasis of cancer cells. Therefore understanding how this gene is regulated is potentially of vital importance to improving cancer management and therapy. Our work has identified a novel transcriptional regulator of survivin called Yin Yang 1 (YY1). YY1 is a transcription factor that has been observed to activate some gene promoters and repress others, and it is gaining increasing interest as a target of cancer therapy. Our work shows for the first time that YY1 is a repressor of survivin transcription and can do so by physically interacting with the survivin promoter. Furthermore, YY1 appears to contribute to basal survivin transcriptional activity, indicating that disruption of its binding may in part contribute to survivin overexpression after cellular stress events including chemo- and radiotherapy. It is also important to use gained mechanistic understandings of cancer initiation and progression to design logical new approaches to cancer therapy. Pancreatic cancer is one of the most deadly forms of cancer known, and survivin expression has been observed to be an important factor in pancreatic cancer aggressiveness or resistance

to therapy. Therefore survivin downregulation may represent an important means of gaining improved treatment efficacy in pancreatic cancer. Using combined gemcitabine and proton radiation therapy, we show that downregulation of survivin and its family member X-linked IAP may lead improved cell death following treatment, particularly when gemcitabine therapy is instituted prior to proton radiotherapy.

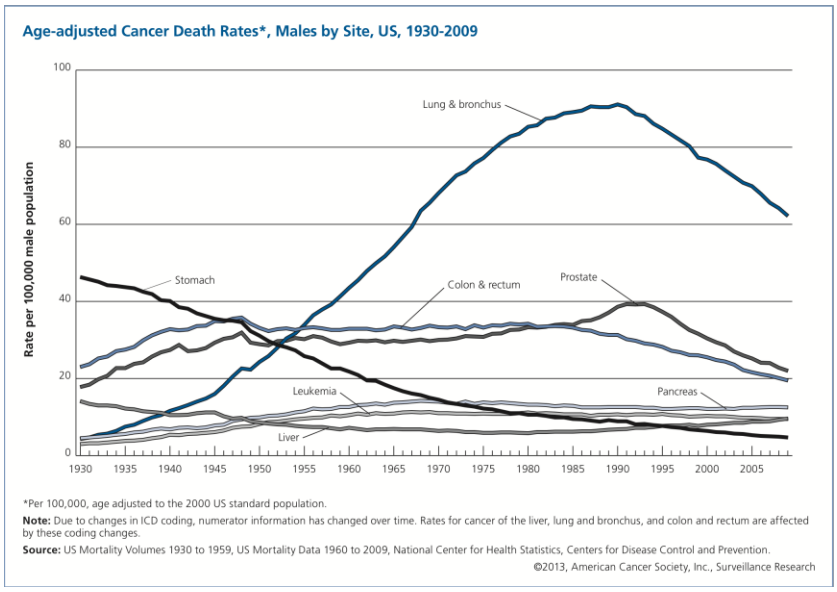
CHAPTER ONE

INTRODUCTION

Cancer Facts and Figures

Cancer is one of the most devastating diseases in the world, and one that has touched the lives of nearly every family and individual in the United States. The National Cancer Institute estimates that in January 2012 there were an estimated 13.7 million individuals living in the United States that had a personal history of cancer. The projected number of new cases of cancer in 2013 is 1,660,290. It is the second most common cause of death, accounting for approximately 1 in 4 deaths in the United States. Furthermore, 580,350 individuals are projected to die as a result of cancer in 2013. Figure 1 illustrates the death rates for males (A) and females (B) from 1930-2009. Fortunately, progress has been made in detection and treatment of cancer, resulting in an increase in overall 5-year cancer survival of 68% between 2001-2008 up from 49% between 1975-1977.

Cancer also imposes an enormous financial burden on the United States. The National Institutes of Health estimate the overall cost of all cancers in 2008 to be $201.5 billion: $77.4 billion for direct medical costs and $124 billion for indirect costs related to premature death and lost productivity[1].



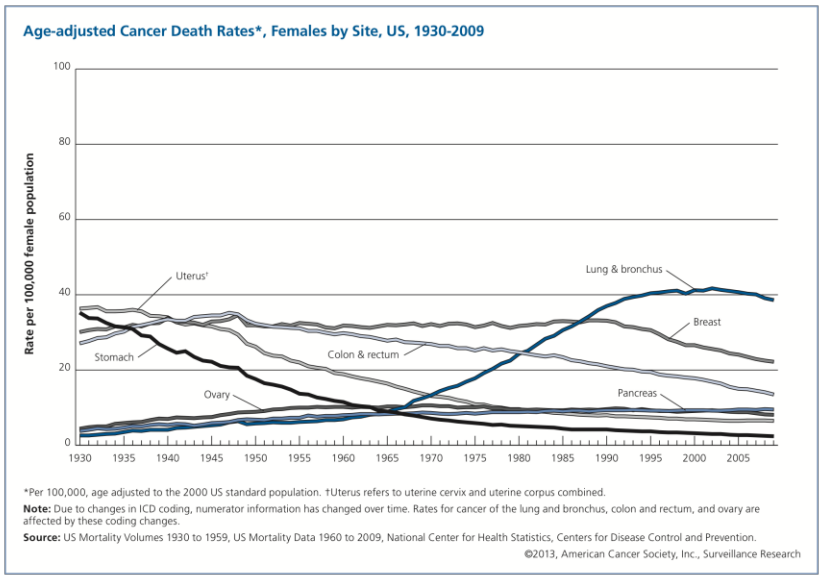


Figure 1. Age-adjusted cancer death rates, 1930-2009. Upper panel shows values for males, lower panel shows values for females. Adapted from American Cancer Society Cancer Facts and Figures 2013 [1].

The leading cause of cancer death in 2013 was lung and bronchus cancer for both men and women. However, prostate and breast cancer continue to have the highest incidence in men and women, respectively. Pancreatic cancer, a disease of particular importance to the chapter 3 of this dissertation, has presented a particularly large challenge to cancer biologists and oncologists, as it continues to be one of the most lethal cancers. Pancreatic cancer will cause an estimated 38,460 deaths in 2013, occuring almost equally in men and women (Figure 2). From 2001-2007, the 5 year survival rate for pancreatic cancer (all stages) was 6%. Since 2004, the overall incidence of pancreatic cancer has been increasing by 1.5% per year.

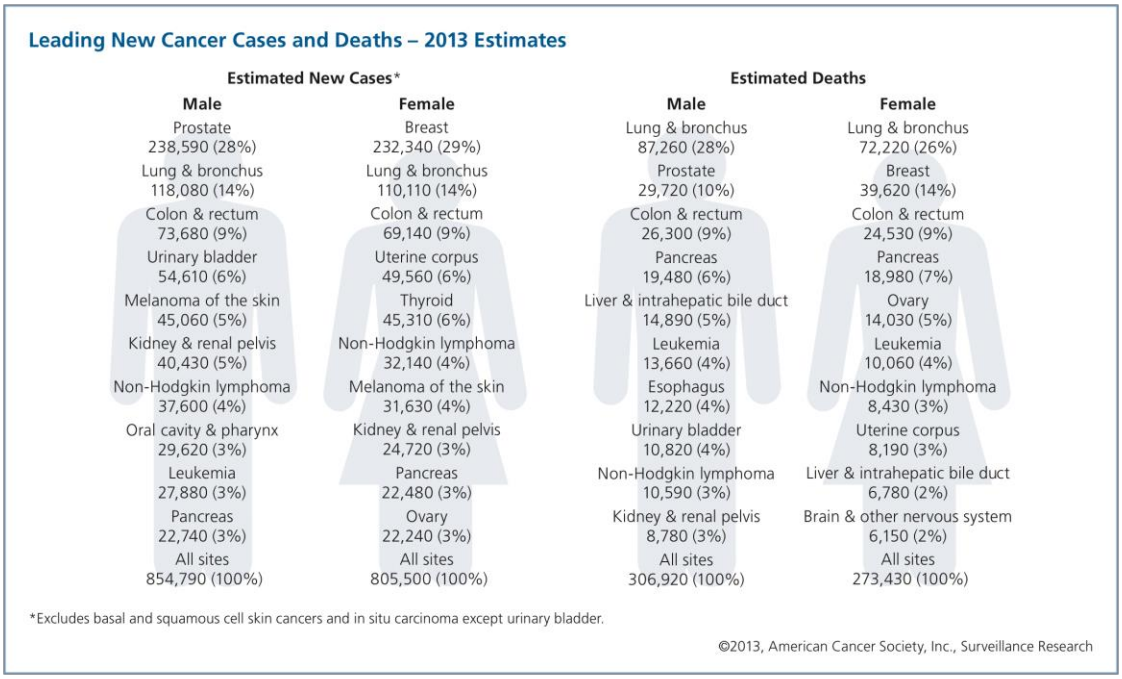


Figure 2. Leading new cancer cases and deaths-2013. The estimated number of new cases for males vs. females is shown of the left, and esimates for cancer deaths in males vs. females is shown on the right. Adapted from American Cancer Society Cancer Facts and Figures 2013 [1].

The figures shown above illustrate that some of the advances made in clinical and basic science are indeed making an impact. However, they simultaneously depict a disease in which scientists may be winning battles, but are clearly still losing the war. This dissertation will explain work that has been done regarding the regulation of a dysregulated cancer gene called survivin that may very well be a key to moving past incremental, small victories in the fight against cancer onto large changes in how cancer of all types is treated, and hopefully one day eradicated. It will also present work that was aimed to take advantage of the James M. Slater Proton Treatment and Research Center facility at Loma Linda University Medical Center by providing evidence for an alternative and potentially more efficacious approach to pancreatic cancer treatment.

The Inhibitor of Apoptosis Survivin

Survivin controls diverse cellular functions including surveillance checkpoints, suppression of cell death, the regulation of mitosis, and the adaptation to unfavorable environments [2-5]. Its suppression of cell death activities and the baculovirus IAP repeat (BIR) domain characterize it as a member of the inhibitor of apoptosis (IAP) family of proteins [6]. However, its lack of a COOH-terminal RING finger domain and the caspase recruitment domain [7] make it structurally unique among the mammalian IAPs. The overall multifaceted functionality of survivin is still being intensely scrutinized, though it appears that protein compartmentalization plays an important role. Survivin has been shown to colocalize in the mitochondria where it abolishes tumor cell apoptosis and promotes tumorigenesis in immunocompromised animals [8]. It, therefore, may possess a role in apoptosis similar to the pro-apoptotic Bcl-2 family of proteins. Survivin has also been found in the nucleus and cytosol where it has roles in mitosis regulation and

apoptosis inhibition, respectively [9]. Survivin has been observed to be expressed in most common human cancers and, while present during embryonic and fetal development, survivin is undetectable in a variety of adult tissues [10]. Its aberrant, high protein expression in cancer cells and concomitantly low expression in most normal tissues makes survivin an important anticancer target [11].

The accumulated data from the characterization of survivin expression in human cancer tissues reveals an overwhelming consistent observation that the expression of survivin is enhanced in various human cancers in comparison with the adjacent normal tissue. Multiple therapeutic strategies have been successfully investigated including the molecular antagonists such as antisense oligos, RNA inhibition, dominant negative mutants, survivin-specific cytolytic T cells, a nonphosphorylatable survivin mutant $Thr^{34™}Ala$ (T34A), and, most recently, binding interface mimetics [12-19]. The observation that a pool of survivin is localized extracellularly and is linked to erosive joint disease in a significant fraction of rheumatoid arthritis patients, and that an autoimmune response (survivin-targeting antibodies) to survivin correlates with protection from joint disease [20,21] provides evidence that anti-survivin therapy may be possible in other pathologies such as cancer. Work in our laboratory is currently defining the role of exosomal survivin in regulation of the tumor microenvironment [22].

While many different therapeutic approaches have been employed, few have been aimed at regulation of survivin transcription. This is owed in large part to the complexity of mechanisms involved in epigenetic, transcriptional, and post-transcriptional gene regulation. It will likely require uncovering of all or most aspects of the machinery

involved in aberrant gene regulation to reach the goal of developing personalized medical approaches to treating unique cancer types.

Survivin Transcription

Survivin transcription is critical in embryogenesis, but is normally turned off in adult life [23]. However, survivin can be transcriptionally upregulated in adult life and often results in disease, particularly cancer. Survivin is the fourth-most frequently overexpressed transcript in most human cancers [24], and the specificity of the survivin promoter for regulation in cancerous tissue has been demonstrated numerous times. It is currently being investigated as a means of driving expression of therapeutic genes [25-27] because its high degree of specificity to malignant cells which could decrease off target expression of a suicide gene or other forms of gene therapy. Survivin's robust and specific upregulation in cancer implies that the transcription factors involved in survivin transcription must be present and themselves upregulated in cancerous tissue. Table 1 summarizes the role of several key transcription factors in survivin transcriptional regulation.

Table 1: Summary of Key Transcriptional Regulators of Survivin (modified from Zhang et al., 2006.

Pathway	Key info and current status
NF-KB	Regulates survivin, but mechanism unclear
p53	Transcriptionally downregulates survivin
APC/Beta catenin/TCF-4	APC dowregulates survivin by inhibiting B-catenin/TCF-4
HIF-1α	Transcriptionally upregulates survivin
Sp1-DNA	Interference of Sp1 interaction-survivin interaction downregulates survivin

Activators of Survivin Transcription

Survivin transcription is induced in part by the presence of cellular stress such as that induced by chemotherapeutic agents, radiotherapy, and aspects of the tumor microenvironment. One such aspect of the tumor microenvironment that is common to most solid tumors is hypoxia and subsequent induction of neovascularization via VEGF and HIF-1α activation. This has lead to investigation a possible relationship between the hypoxia-responsive gene HIF-1α and survivin. A study from Wei et al. [28] found a strong correlation between HIF-1α and survivin expression in immunohistochemically analyzed pancreatic cancer samples. Follow up studies found that use of antisense HIF-1α in pancreatic cancer BxPc-3 cells inhibited survivin expression and induced apoptosis in cells [29]. Peng et al. found an association between Epidermal Growth Factor (EGF) overexpression and survivin overexpression [30]. This EGF-related upregulation was mediated by HIF-1α transcriptional activation of the survivin gene, even under normoxic conditions. Bai et al. more recently identified a strong relationship between survivin overexpression and HIF-1α overexpression in cervical cancer [31]. They show HIF-1α-responsive element independent upregulation of survivin reporter constructs, specifically in the first 158 bp of the survivin promoter. Indeed HIF-1α-mediated upregulation of survivin has now been observed in many cancer types including pancreatic, prostate [32], cervical [31], non-small cell lung [33], laryngeal [34], and colorectal cancer [35]. Efforts are underway to evaluate the effectiveness of disruption of HIF-1α expression as a means to sensitize cells to therapeutic modalities.

It is now known that basal survivin transcription requires Sp1 (more will be said about Sp1 later in this dissertation) or KLF5 [36,37], but there are numerous other

transcription factors and coactivators that are thought to drive high levels of survivin transcription. NF-κB is one of these transcription factors. It is a complex of proteins that are involved in inflammation, increased cell proliferation, metastasis, and inhibition of apoptosis. One of the mechanisms by which it contributes to these phenotypes is by transcriptional activation of survivin [38-40]. Members of the signal transducers and activators of transcription (STAT) family are also transcription factors capable of binding and activating the survivin promoter [41].

Downregulation of Survivin Transcription

Several key proteins are also able to downregulate survivin transcription. In addition to p53's critical involvement in cell cycle checkpoint regulation, it also prevents the transcription of oncogenes such as survivin. Retinoblastoma (Rb) and E2F have similar effects on survivin transcription [42]. However, these genes are often silenced, mutated, and/or nonfunctional in patients with cancer. Therefore identification of other transcription factors that may negatively regulate survivin is of importance to cancer therapy. Egr-1, a transcription factor that shares many similarities with YY1, has previously been noted to be involved in cell cycle, death, and differentiation. Much like YY1, Egr-1 can either act as an activator or repressor depending on the promoter in question and the available coregulators with which it can interact. Egr-1 has a consensus binding site that shares some overlap with the Sp1 transcription factor [43,44]. Interestingly, YY1 can also be repressive or activating depending on a number of factors, and it also shares some overlap with Sp1 binding sites for some of its targets [45]. This dissertation will show that the transcription factor YY1 may be involved in direct transcriptional repression of survivin, which may reveal a novel means of studying inhibition of survivin

transcription for therapeutic treatments.

Natural agents are gaining increasing interest as a means of distrupting oncogene transcription, including survivin. YM155, a small molecule inhibitor of survivin, has recently been investigated in Phase II clinical trials for a variety of cancers including diffuse large B-cell lymphoma [46], prostate cancer [47], melanoma [48], and non-small cell lung cancer [49] due to its previously observed ability to induce apoptosis and reduce tumor bulk in various *in vitro* and *in vivo* models [50]. This induction of cell death is thought to be due, at least in part, to its ability to decrease survivin transcription, but the mechanism by which it does this is still under investigation. Nakamura et al. [51] recently found a role for interleukin enhancer-binding factor 3 (ILF3/NF110) in this observed inhibition of survivin expression by YM155. They also found that in luciferase reporter experiments, ILF3-dependent upregulation of reporter activity could be attenuated with YM155, suggesting that ILF3/NF110 is a physiological target of YM155. Currently, clinical trials are showing promise for YM155, particularly as a combination therapy to sensitize tumors to existing therapies. Other natural agents are also showing potential for disruption of survivin transcriptional activity. Specificity proteins Sp1, Sp3, and Sp4 have long been known to be important transcription factors involved in the overexpression of survivin in human cancer. However, little progress has been made to exploit this relationship for gains in therapeutic approaches to cancer. Recently, curcumin was identified as a natural agent that inhibits the ability of Sp1, Sp3, and Sp4 to activate survivin transcription [52]. It appears to do so by generating reactive oxygen species that upregulate repressors of Sp proteins ZBTB10 and ZBTB4, and downregulation of the microRNAs mir-20a, mir-27a, and mir-17-5p, that are regulators

of these Sp repressors. Interestingly, curcumin is also showing promise as a sensitizing agent to ionizing radiation in Burkitt lymphoma and non-Hodgkin lymphoma [53,54]. The natural agents Resveratrol and Quercetin in combination (RQ) have also shown a similar downregulation of Sp proteins and their targets, including survivin [55]. Interestingly, the authors cite RQ's antioxidant capabilities (as opposed to curcumin's generation of reactive oxygen species) as the potential reason for this observed repression of Sp protein and their transcriptional targets such as survivin. These data further support the need for continued efforts to develop therapeutic approaches to cancer that include disruption of survivin transcriptional activation.

The Multifunctional Transcriptional Factor Yin Yang-1

Yin Yang-1 (YY1) is a 65-kDa ubiquitous multifunction transcription factor that is a member of the GLI-Kruppel family of nuclear proteins [56-58]. This family of proteins plays roles in development and exerts much of its function through cell cycle regulation. YY1 is a relatively unique transcription factor in that it can act by repressing some genes and activate others by binding to the specific DNA sequence 5'-CGCCATNTT-3' [57,59]. This phenomenon was noted first when it was shown that YY1, in the presence of the adenovirus-derived protein called E1A, represses the AAV P5 promoter [60]. When E1A is not present, YY1 then activates transcription [61].

Reports suggest that YY1 is required for cell survival, as complete ablation of YY1 results in lethality [62]. Furthermore, array data suggests that YY1 has roles in cell cycle, cell adhesion, and other markers of disease aggressiveness [63,64]. As is true for survivin, YY1 is increasingly found to be involved in cell death regulation via NF-κB. Within the serum amyloid A gene, there is a binding site for NF-κB that was found to

overlap with a YY1 binding motif. Lu et al. showed that YY1 binding was able to abrogate NF-κB binding and transcriptional activity. A similar binding site overlap was observed in a cytomegalovirus promoter [65]. This offers some indirect evidence of YY1 involvement in cell death, but more direct evidence is also emerging. Evidence suggests that YY1 transcriptionally represses Fas, which in turn means that YY1 is a significant factor in resistance to Fas-induced apoptosis [66]. YY1 also appears to have a direct role in resistance to tumor necrosis factor-related apoptosis inducing ligand (TRAIL). Recent findings show a direct role for YY1 negatively regulating transcription of death receptor 5 (DR5), meaning YY1 is also a resistance factor for TRAIL-induced apoptosis [67].

YY1's Role in Human Cancer

YY1 is gaining increasing interest as a cancer-related transcription factor. The oncogenic role of YY1 has been reviewed numerous times [68-70], yet many questions remain. Consistent with its variable role as a transcription factor depending on a multitude of cellular and molecular conditions, it appears to have a variable role in cancer depending on what type of cancer is being studied. Intriguingly, YY1's role in some cancers appears to promote longer patient survival, whereas in others it correlates with poorer outcomes and shorter survival. Table 2 summarizes current findings regarding YY1's role in various cancer types. A computational analysis of YY1 expression in numerous data sets that looked at a broad array of cancer types indicates a relative increase in YY1 expression compared to expression in normal tissue. Seligson et al. have shown that YY1 protein levels are higher in metastatic prostate cancer tissue than in primary tumor. However, they also found a correlation with lower YY1 protein levels and survival, suggesting that lower YY1 levels may lend a survival advantage to

metastatic cells [71]. Further supporting a role for YY1 in prostate cancer formation, Deng et al. found that in prostate cancer YY1 interacts with androgen receptor (AR) to promote PSA transcription [72].

A similar association of YY1 with disease progression has been noted in intraepithelial neoplasms and cervical cancer. YY1 expression in high-grade squamous intraepithelial lesions is higher than in low-grade squamous intraepithelial lesions, a finding also consistent with the observation that high expression correlates with the presence of Human Papilloma Virus infection [73].

There are also many reports of a direct role for YY1 in aberrant cell cycle in cancer. Numerous studies show that YY1 is involved in tumorigenesis via interactions with the tumor suppressor p53. The general mechanism it appears to do this by is interference of p53-dependent transcription of its target genes by competing for binding to the ACAT sequence of promoters [74]. Also, YY1 has been shown to be essential for optimal interaction of MDM-2 and p53, which is required for MDM-2 ubiquitination of p53 [75]. The importance of this finding cannot be overstated, as an estimated 50% of all tumors have p53-inactivating mutations [76].

Table 2: YY1 expression in human cancers and it's clinical relevance (modified from Castellano et al. [68]).

Tumor Type	Methods	Clinical relevance of YY1 Overexpression	Reference
Prostate cancer	IHC	Positive correlation with metastasis and inverse relationship with poor outcome	***71***
Ovarian cancer	Microarray	Positive correlation with long-term survival	***77***
Ovarian c`ancer	Microarray, IHC, RT-PCR	Positive correlation with survival and response to taxanes	***78***
Cervical neoplasms	RT-PCR	Positive correlation with disease progression	***73***
Osteosarcoma	RT-PCR, IHC, WB	Positive correlation with more malignant phenotype	***79***
Myeloid Leukemia	RT-PCR	Positive correlation with t(8;21)	***80***
Non-Hodgkin Lymphoma	RT-PCR	Positive correlation with poor outcome	***81***
Follicular Lymphoma	IHC	Positive correlation with length of survival	***82***

Pancreatic Cancer: Toward Improved Combination Therapy

Pancreatic cancer is the fourth leading cause of cancer-related death (refer to Figure 2). Early detection of localized disease with subsequent surgical resection offers virtually the only hope of long-term survival to pancreatic cancer patients. Unfortunately, this represents only an estimated 10-20% of all patients. Because chemotherapy has offered very minimal improvements in survival time, efforts to use radiation in combination have been explored and been met with some success [83]. Doses for radiation therapy are limited, however, by the proximity of other structures that are subject to bystander toxicity such as the liver, kidneys, stomach, spinal cord, and small intestines. Proton radiotherapy is a powerful means of treating cancer as it offers the advantage of allowing delivery of a given radiation dose at the depth of a tumor, but not beyond. Thus, non-tumor tissue beyond the tumor is spared from radiation and the long-term complications of such exposure. If off-target damage can be reduced, the dose of radiation used on the tumor can be increased to improve efficacy of the treatment. Unfortunately, tumors can develop radioresistance due to changes in molecular determinants of cell death.

Reports suggest that survivin is one factor involved in imparting radioresistance to tumors. In a study of pancreatic duct cell carcinoma (PDC) vs. precancerous intraductal papillary-mucinous tumor (IPMT), Satoh et al. found high survivin expression in PDC as opposed to very low expression in IPMT, suggesting that survivin is involved in the progression to a malignant phenotype in pancreatic cancer [84]. A second facet of this dissertation is exploration of combined therapy with gemcitabine and proton therapy. Currently, proton therapy is used mostly for prostate, brain, and head and neck cancer. This is largely due to ease of targeting these structures. However, efforts are underway to

evaluate the effectiveness of proton therapy for pancreatic cancer. A Phase II clinical trial at Loma Linda University Medical Center is currently exploring the role of proton therapy in combination with different chemotherapy regimens in locally advanced pancreatic cancer treatment. In addition to advancing the understanding of potential advantages of proton therapy in pancreatic cancer cell death, the work presented in Chapter 3 presents evidence that survivin and its IAP family member X-linked IAP (XIAP) may be key molecular determinants of apoptosis and radioresistance in pancreatic cancer. If a viable means of modulating survivin and XIAP levels in a clinical setting is discovered, this may offer a means of drastically improving response to therapy.

Design of Studies

The studies presented in the chapters to follow were designed to advance the understanding of both basic science aspects of cancer biology and provide data to improve the therapeutic approach to pancreatic cancer. Most effective cancer therapies are based on a sound rationale that was developed from basic science research to discover molecular and cellular behavior after manipulations, whether they are genetic, medicinal, or immunological. Chapter 2 of this dissertation details a study of transcriptional regulation of survivin in attempt to better understand factors involved in survivin overexpression in cancer. To do this we used a luciferase reporter system that allows one to study promoter activity in the presence of modifying factors including cellular stress and transcription factor overexpression. Furthermore, we used electrophoretic mobility shift assays (EMSA) to determine whether or not YY1 was capable of binding directly to the survivin promoter. This is an important step to try to establish how a

transcription factor is affecting target gene expression because it can do so by many means aside from direct promoter binding.

In Chapter 3, the goal was to discover if different doses and timing of a combination of gemcitabine and proton therapy could sensitize pancreatic cancer cell to enhanced cell death, and to see if this enhanced cell death correlated with survivin expression. To study cell death after each treatment combination, we used propidium iodide flow cytometry and western blots to investigate the concomitant survivin expression. We also chose to compare a radiosensitive cell line (MiaPaCa-2) with a radioresistant cell line (Panc-1) to better define the usefulness of proton therapy in our treatment schemes.

These studies, done in cell culture models, were designed to establish preclinical rationales for later work to be done in animal models, or in the case of Chapter 3, patients. As previously mentioned, efforts to develop therapeutic approaches based on transcription factor modulation are already underway, and clinical trials using proton therapy for pancreatic cancer are underway as well, including a Phase II clinical trial at Loma Linda University Medical Center.

Importance of Studies

The advent of molecular biology has given scientists powerful tools to understand the mechanisms and architecture involved in cell structure and function, and has helped reveal the true complexity of biological systems. A key feature of this complexity is redundancy, a concept that has plagued therapeutic approaches to cancer. Molecular biology has revealed that virtually no cellular processes are without pathway redundancy, and cancer cells have perhaps even more redundancy than normal cells to better equip

them to evade immune response and death. Gene expression, such as that of survivin, is affected by redundant epigenetic, transcriptional, and post-transcriptional regulation factors. In order to most effectively exploit therapy directed against a target such as survivin, it is important to understand the complete picture of how the survivin gene works. This dissertation will detail what we believe is a novel regulator of survivin transcription, YY1. Specifically, YY1 may be a negative regulator of survivin and may be the focus of therapeutic approaches to cancer therapy in the near future.

In addition to obtaining a more complete understanding of molecular mechanims involved in oncogenesis and tumor suppression, it is critical to continue to design new therapeutic approaches based on sound reasoning arrived at through basic science research. This dissertation also details the use of combined chemotherapy and proton radiation therapy as a means to treating pancreatic cancer. Since our work began, clinical trials utilizing combined gemcitabine and proton therapy have been conducted in Japan, and are showing great promise [85]. One of the key limiting factors in radiation treatment is organ-related or systemic toxicity. Proton therapy is an extremely effective means of delivery radiation while simultaneously sparing surrounding non-malignant tissue compared to standard gamma or x-ray radiation [86,87]. Currently, proton therapy is not used as a means of treating pancreatic cancer at Loma Linda Medical Center, but the work presented in Chapter 3 provides evidence that may stimulate more thought as to whether or not that should change.

CHAPTER TWO

YIN YANG 1 REGULATES THE TRANSCRIPTIONAL REPRESSION OF SURVIVIN

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Running Title: Survivin's regulated transcriptional activity

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Abstract

Survivin is a member of the Inhibitor of Apoptosis (IAP) family of proteins, and is highly expressed in all cancers but absent in most non-proliferative normal tissue. Expression level correlates with chemo- and radioresistance, as well as poor prognosis in cancer patients. The mechanisms for upregulation of survivin in cells undergoing stress associated with tumor development and the tumor microenvironment are not well understood. The putative stress response transcription factors HIF-1α and Yin Yang 1 (YY1) were hypothesized to contribute to the upregulation of Survivin in tumor cells. Examination of the 5' flanking region of human survivin gene revealed the presence of multiple putative stress activated transcription factor binding domains that have previously been shown to be associated with HIF-1α and YY1. In order to study basal regulation with luciferase reporter assays, U2OS cells were transfected with a variety of constructs of the survivin promoter. As expected, cells overexpressing HIF-1α showed a 2-3 fold transactivation of all promoter constructs tested. Surprisingly, when YY1 is overexpressed in this survivin promoter reporter system, luciferase expression was repressed 30-40 fold. YY1 involvement in survivin promoter repression was confirmed using siRNA directed against YY1. These studies showed that knockdown of YY1 releases the survivin promoter from the observed repression and leads to a 3-5 fold increase in promoter activity above basal levels. A U2OS cell line containing a stable YY1 Tet-off system was used to determine whether a temporal increase in YY1 expression affects Survivin protein levels. A low to moderate decrease in Survivin protein was observed 24 hrs and 48 hrs after Tet removal. Studies also confirmed that YY1 is capable of directly binding to the survivin promoter. Collectively, these findings

identify novel basal transcriptional requirements of survivin gene expression. While HIF-1α may be in part responsible for the increased expression of survivin in tumor tissue, YY1 may also be induced under stressful conditions to negatively regulate survivin, suggesting that it is the balance of these transcription factors, and likely others, that may play an important role in the development of cancer and resistance to its treatment.

Introduction

Survivin, a unique mammalian inhibitor of apoptosis (IAP) protein, controls stress from the microenvironment through diverse functions within the cell including surveillance checkpoints, suppression of cell death, regulation of mitosis, and adaptation to unfavorable environments [2-4]. Unlike all other IAP family members, survivin is unique in that it has important regulatory roles in both apoptosis suppression and cell cycle progression regulation [88]. Survivin has been observed expressed in most common human cancers and present during embryonic and fetal development [10]. Its aberrant, high protein expression in cancer cells and concomitantly low expression in most normal tissues makes survivin an important anticancer target [11]. Strategies have been successfully investigated against survivin, including molecular antagonists such as antisense oligos, RNA inhibition, ribozymes, dominant negative mutants, survivin-specific cytolytic T cells, a nonphosphorylatable survivin mutant Thr^{34}™Ala (T34A), triplex DNA formation and most recently, binding interface mimetics [12-19,89-92].

Epigenetic, genetic and post-translational mechanisms for survivin gene regulation have been described in many malignant cell types [42] with various transcription

factors including Stat3 [41], HIF-1α [30], Rb-E2F1 [93], Dec1 [94], Sp1 [36], c-myc [95] and KLF5 [37] affecting its transcriptional upregulation. In addition, the tumor suppressor p53 and Rb-E2F2 have been shown to repress survivin transcription by direct binding to the survivin promoter in a lung adenocarcinoma cell line [96] and in normal human melanocytes [42]. Survivin's promoter has been recorded to be differentially methylated in ovarian cancers as compared to normal ovarian tissues. An interesting polymorphism has also been described at a CDE/CHR repressor element in the survivin promoter that correlates with increased survivin mRNA and protein in cancer cell lines and not in normal cell line controls [97].

The transcription factor YY1 is known to have a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation [61]. YY1 exerts its effects on genes involved in these processes via its ability to initiate, activate, or repress transcription depending upon the context or recruited cofactors in which it binds [98,99]. One such family of cofactors are the histone deacetylases which have been shown to bind YY1 and repress transcription when targeted to promoters [100]. YY1 has been shown to interact with p300, PCAF and CBP, all which posses the histone acetyltransferase (HAT) activity [100]. YY1 may thus activate transcription by its recruitment of HAT proteins and repress trancription by recruiting HDACs.

Poor oxygenation (hypoxia), owing to an inadequate blood supply, is a common feature of most solid human tumors and is associated with increased malignancy, resistance to therapy and distant metastasis [101]. Hypoxia inducible factor-1α (HIF-1α), a member of basic helix-loop-helix-PAS protein family [102,103], is usually increased under

hypoxic conditions, and can activate transcription of many genes that are critical for cellular function under hypoxic conditions [102]. Previous studies have found that down-regulation of HIF-1α could significantly decrease the levels of survivin expression in BxPc-3 pancreatic cancer cells [29] and breast cancer cells [30]. HIF-1α was also demonstrated to directly bind to the survivin promoter, which strongly suggests that survivin gene expression is indeed mediated by HIF-1α under normoxic conditions [30].

In the present study, we examined the transcriptional affect of YY1 and HIF-1α on survivin in an osteosarcoma cell line derived from human bone osteosarcoma (U2OS). We found that when YY1 and HIF-1α were overexpressed in U2OS cells, survivin mRNA and protein were repressed by YY1 and induced by HIF-1α. By analyzing the survivin promoter activity, we further found that YY1 was a transcriptional repressor of the survivin gene while HIF-1α was a transcriptional activator. We also show, for the first time, that YY1 is capable of binding directly to the survivin core promoter and thus is acting as a transcription factor rather than an corepressor.

Results

Identification of Survivin Promoter Sites Involved in Transcriptional Regulation by HIF-1α and YY1

HIF-1α has previously been shown to be a transcriptional regulator of survivin [28,30,31]. To determine possible bindings sites for YY1, using a computer-based approach the survivin promoter was scanned for putative HIF-1α and YY1 binding sites using the online tool TFSearch (www.cbrc.jp/research/db/TFSEARCH.html) and previously published consensus sequences [104]. Figure 1 shows the locations of all identified HIF-1α and YY1 consensus sites in the first 6280 bp of the survivin promoter. Using Survivin

promoter nested deletions in a luciferase reporter system (previously described by Li and Altieri [105]), the ability of YY1 and HIF-1 to activate or repress survivin promoter activity was tested (Fig 2. A). The survivin promoter nested deletions were utilized to assist in identification of regions of the promoter that are essential to regulation of the survivin promoter by each transcription factor tested. Therefore constructs ranging from 230 bp upstream of the surviving start site up to 6280 bp upstream of the start site were utilized. When HIF-1α was overexpressed in U2OS cells, an induction of 2-3 fold was seen in all constructs tested except +230 bp and +6280 bp. However, when YY1 was overexpressed, contrary to our initial hypothesis, there was a 30-40 fold repression of survivin promoter activity in all constructs tested. To further examine these findings, we evaluated endogenous survivin transcript levels after overexpression of HIF-1α and YY1 in U2OS cells (Fig. 2B). The results were consistent with survivin transcriptional upregulation by HIF-1α as seen in the previous reporter experiments, and downregulation of survivin after YY1 overexpression.

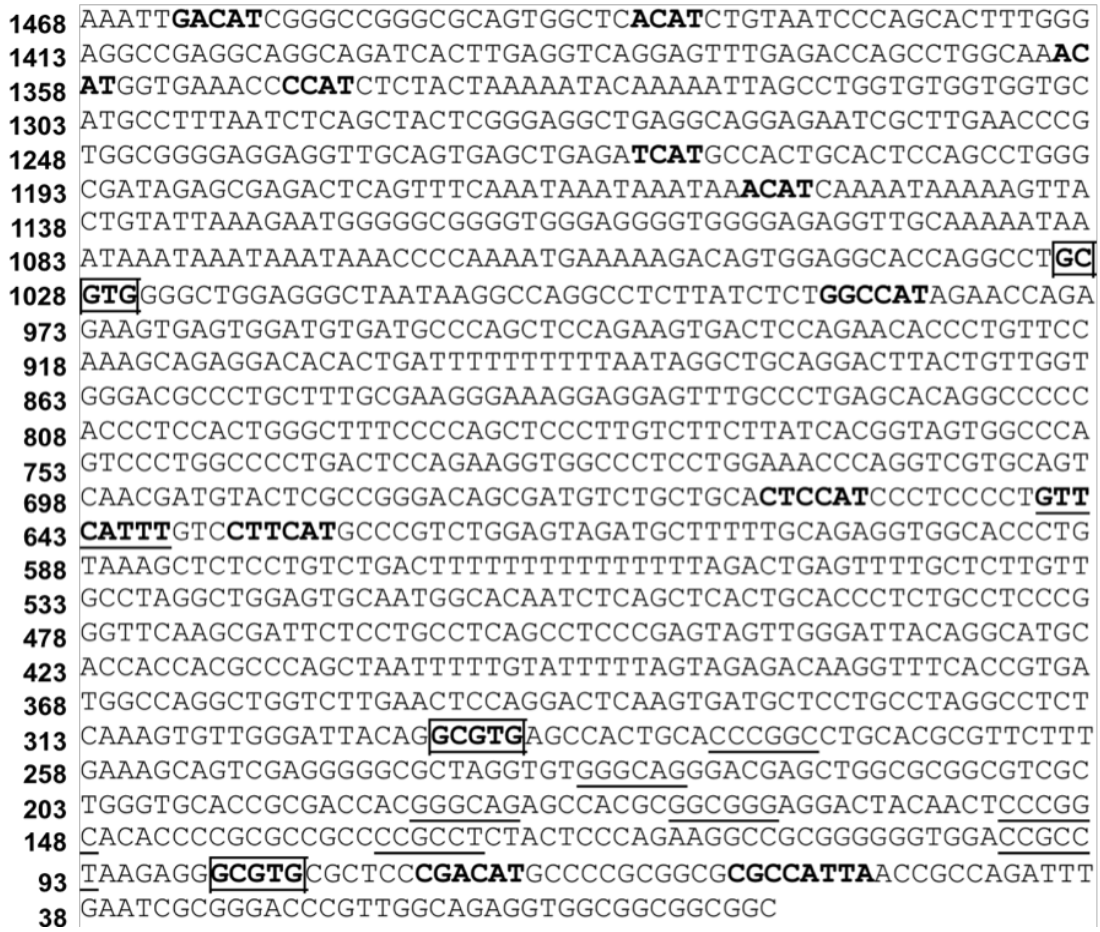
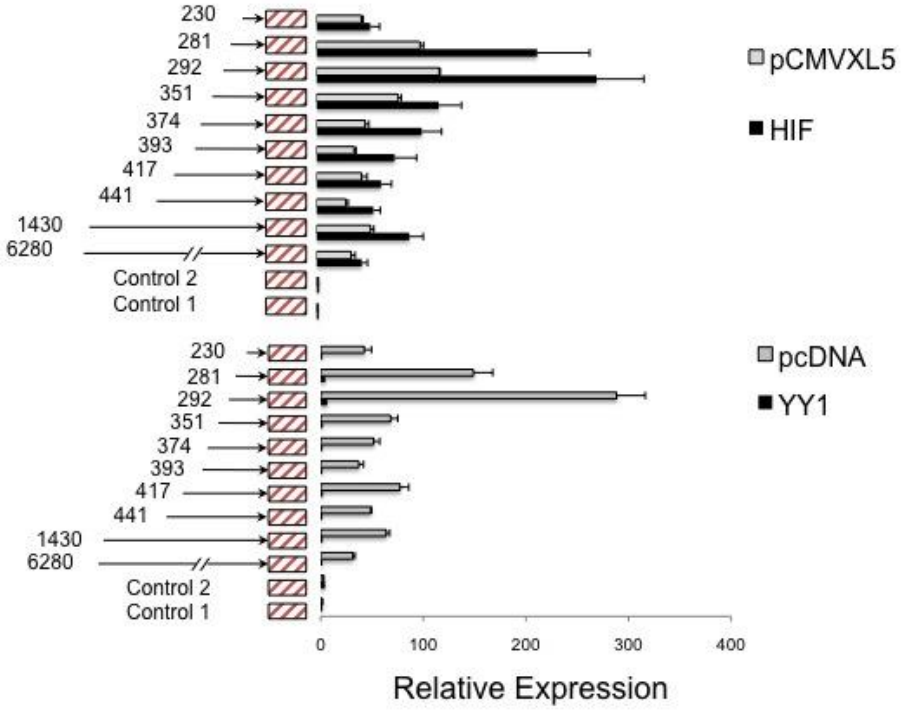
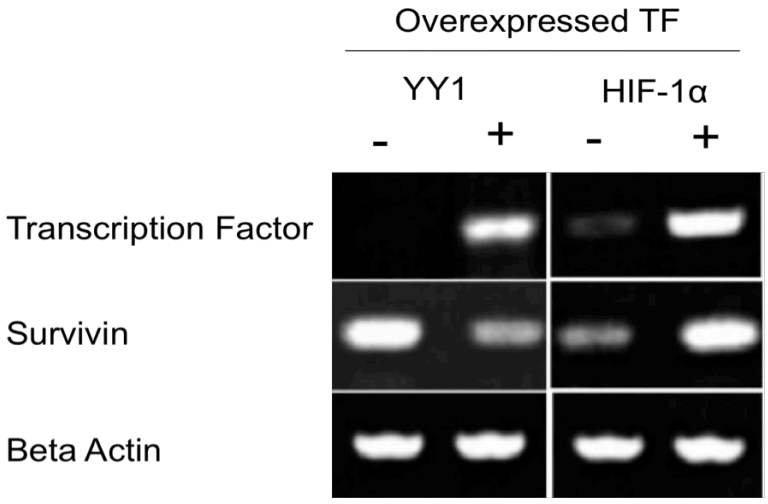


Figure 1. Proximal survivin promoter schematic. Using previously published putative DNA binding sites for YY1, HIF-1, and TFSearch, the survivin promoter was scanned for the presence of each of these putative binding sites. Analysis revealed the presence of multiple putative YY1 binding sites, noted by bolded segments. Similarly, analysis of the survivin promoter revealed several putative HIF-1 binding sites (noted as the boxed segments). For reference, putative SP1 sites are also denoted as underlined segments.

Figure 2. Effect of HIF-1 and YY1 overexpression on survivin promoter activity and transcript levels. (A) Luciferase reporter assays were performed using survivin promoter constructs in the pGL3Basic vector ranging in length from +6280 bp to +230 bp. U2OS cells were transiently cotransfected with survivin construct DNA of the indicated length, and either YY1, HIF-1, or their respective empty vector for baseline promoter activity. Controls indicate relative values of expression when empty pGL3 was contranfected with empty transcription factor expression vector (Control 1) or empty pGL3 contransfection with the indicated transcription factor (Control 2). Error bars represent the standard deviation of duplicate luminescence measurement. Results are representative of repeat experiments. (B) RT-PCR analysis of survivin expression following overexpression of YY1 and HIF-1. A (-) indicates that cells were transfected with the corresponding empty vector for each transcription factor. Beta actin is shown for reference as a loading control.


230
281
292
351
374
393
417
441
1430
6280
Control 2
Control 1
pCMVXL5
HIF
pcDNA
YY1
0
100
200
300
400
Relative Expression

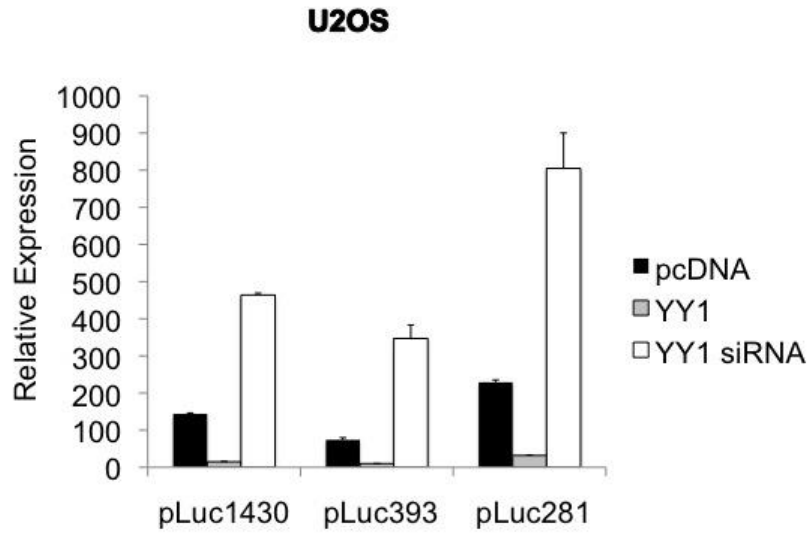

Overexpressed TF
YY1
HIF-1α
-
+
-
+
Transcription Factor
Survivin
Beta Actin

siRNA-mediated Knockdown of YY1

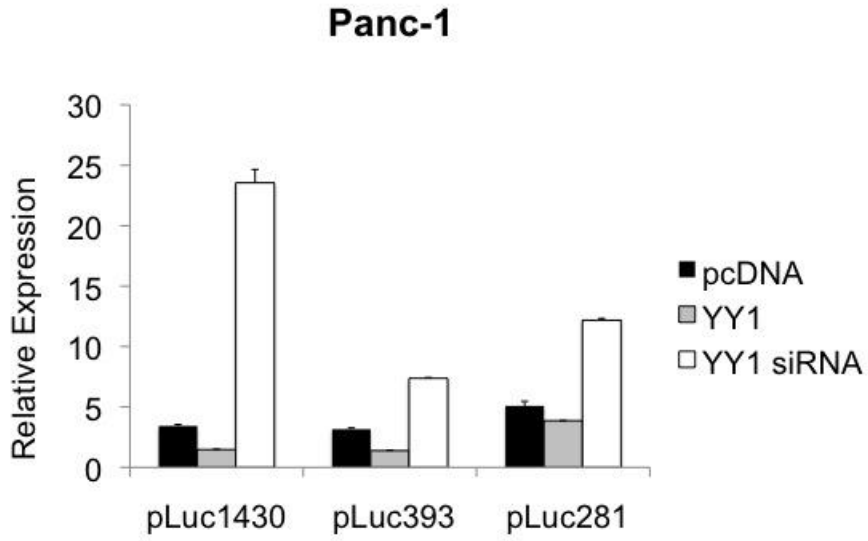
Due to the unexpected and robust findings for YY1, it was chosen for further studies. To provide further evidence for the ability of YY1 to affect basal survivin promoter activity, YY1 knockdown was performed (Fig. 3 A and B). Because previous experiments showed survivin promoter activity repression across all constructs tested, pLuc1430, 393, and 281 were chosen for this experiment. In U2OS (Fig. 3A) and Panc-1 (Fig. 3B) cells alike, siRNA knockdown of YY1 relieved the survivin promoter of basal promoter activity repression, indicated by an increased in luciferase reporter activity of approximately 3-4 fold in each construct tested. The overexpression of YY1 again repressed promoter activity to nearly undetectable levels, a finding consistent with previously described experiments.

Figure 3. YY1 siRNA relieves the survivin promoter from transcriptional repression. Luciferase reporter assays were performed after YY1 overexpression and siRNA knockdown in (A) U2OS and (B) Panc-1 cells. Three survivin promoter reporter constructs were tested (pluc1430, pLuc 393, and pLuc 281). Relative expression indicates promoter activity relative to luciferase activity in the presence of empty pGL3 vector and background pRL-tk activity. Error bars represent the standard deviation of duplicate luminence measurements. Results are representative of repeated experiments.

A.


U2OS
Relative Expression
1000
900
800
700
600
500
400
300
200
100
0
pLuc1430
pLuc393
pLuc281
pcDNA
YY1
YY1 siRNA

B.


Panc-1
Relative Expression
30
25
20
15
10
5
0
pLuc1430
pLuc393
pLuc281
pcDNA
YY1
YY1 siRNA

Protein Expression of Survivin is Modulated by YY1 Overexpression

To investigate whether YY1 expression can affect survivin expression at the protein level, Western blot analysis was done using a U2OS YY1 tet-off cell line (previously described by Sui et al. [75]). Twenty-four hours after tet removal, a significant YY1 overexpression was seen (Figure 4). Survivin protein levels remained unchanged at 24 hours. However, after 48 hours of incubation in tet-free media, a modest reduction in Survivin protein expression was seen.

Site-directed Mutagenesis of Putative YY1 Bindings Sites in the Survivin Promoter

Repression of survivin promoter activity in our luciferase reporter system was seen in all constructs tested, including the shortest construct containing 230 bp of the promoter, which has previously been shown to be the core promoter for survivin. Fig. 1 illustrates two putative YY1 binding sites within the first 230 bp of the survivin promoter, and we therefore wanted to investigate the involvement of these two sites as key areas involved in repression of basal survivin transcription by YY1. Site-directed mutagenesis was employed to define the role of these two sites in survivin transcription. Fig. 5A illustrates the mutation of each CAT region of the putative YY1 sites to GGG. This region was chosen for mutation based on previous studies indicating that the core sequence preferred by YY1 is CCAT or ACAT [104]. When both putative YY1 binding sites were mutated, neither overexpression or knockdown of YY1 are able to alter the basal survivin promoter activity (Fig. 5B). Furthermore, the basal survivin promoter activity (pcDNA empty vector only) was increased relative to non-mutated promoter

activity. These data support a role for these putative YY1 binding sites in basal survivin transcriptional activity.

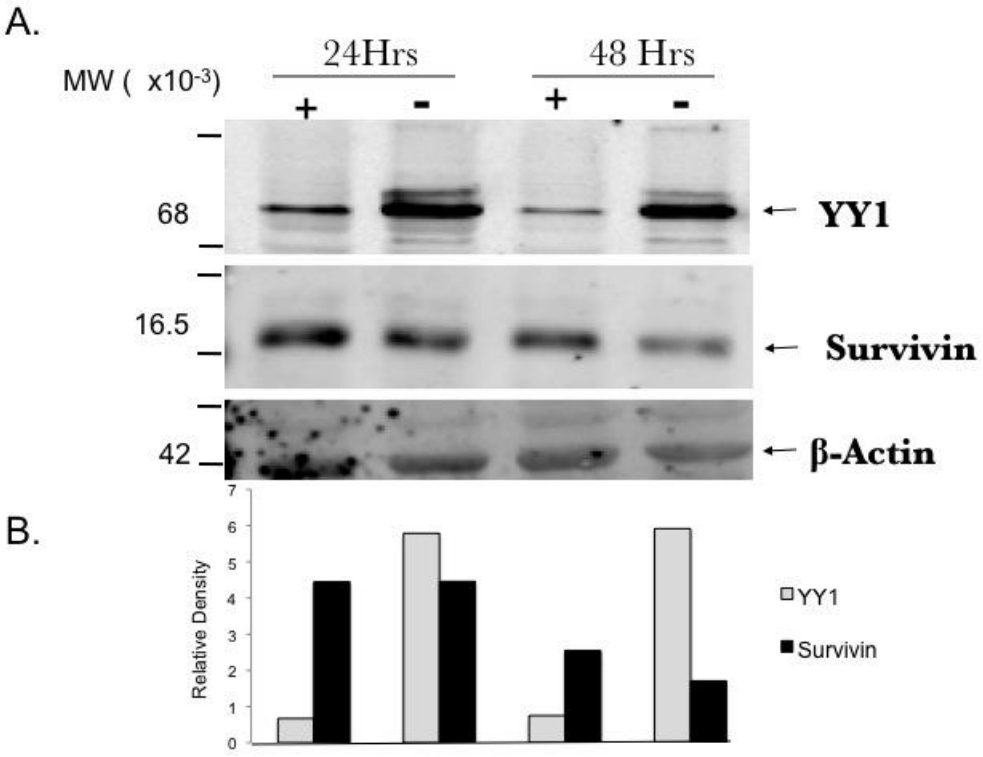


Figure 4. Survivin expression decreases after 48 hours of YY1 overexpression. Western blot analysis of survivin expression after YY1 overexpressionin via tet-off U2OS cells was analyzed. A) U2OS cells that stably express a YY1 overexpression vector under the control of a tetracycline responsive promoter were cultured to 70-80% confluency in the presence of 0.1 mg doxycycline. They were then washed 7-8 times in tet-free media and cultured for either 24 or 48 hours in tet free media. They were then lysed and protein was extracted for western blot analysis. +/- indicates the presence or absence, respectively, of tet in the culture media. (B) Densitometric analysis of Western blot bands. Bars represent density of YY1 (light bars) or survivin (dark bars) normalized to beta actin band density.

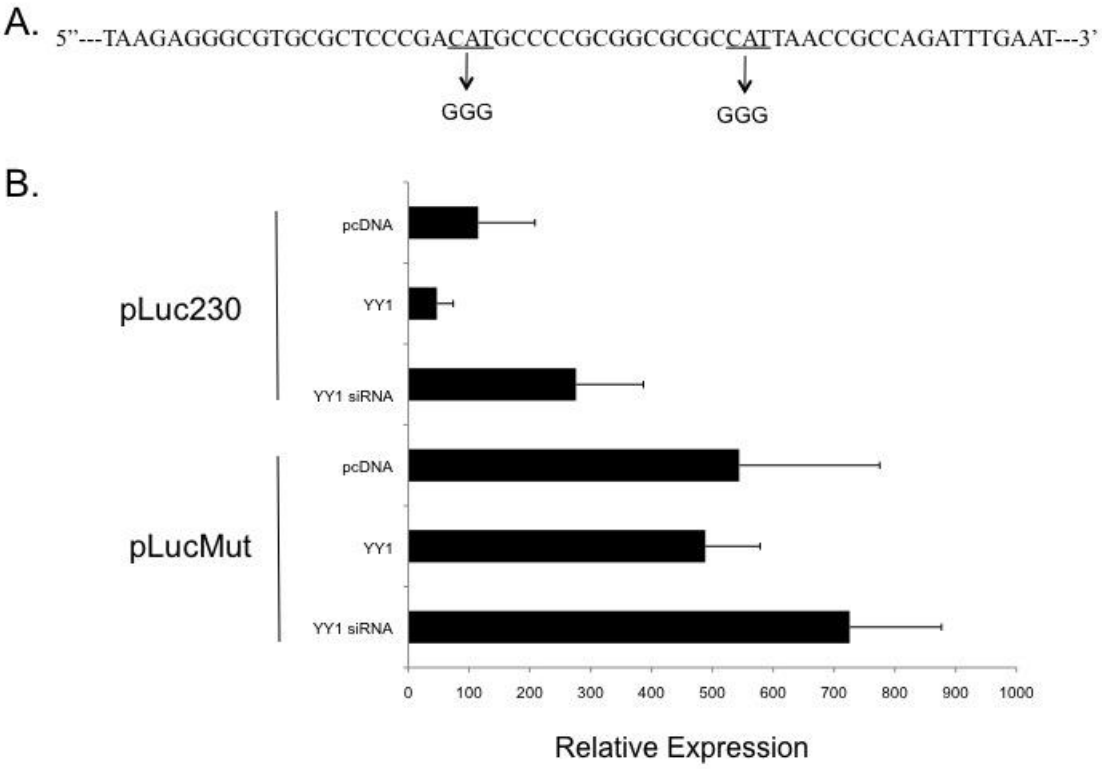


Figure 5. Mutation of two putative YY1 binding sites in the proximal survivin promoter alters promoter activity (A) The two most proximal putative YY1 binding sites (contained within pLuc230 construct) were mutated from the core YY1 recognition site CAT to GGG. (B) Luciferase reporter assay. U2OS cells were transfected with either (1) pLuc230, the standard pGL3 vector containing 230 unmutated bp of the survivin promoter, or (2) pLucMut in which the two putative YY1 binding sites were mutated from CAT to GGG. Each vector was cotransfected with either empty pcDNA, YY1, or YY1 siRNA as well as pRL-tk for transfection efficiency internal control. Error bars represent standard deviation of duplicate luminescence measurement, and results are representative of multiple experiments.

YY1's Interaction with the Core Survivin Promoter

YY1 can exert transcriptional changes via direct DNA binding or through protein-protein interaction. In order to determine if the survivin repression seen in reporter assays, RT-PCR, and Western blotting is through direct binding of YY1 to the survivin promoter at locations identified in Fig. 1, electrophoretic mobility shift assay was performed. Two putative YY1 binding sites located in the survivin core promoter (Fig. 6A) were studied. To validate the study, a YY1 consensus sequence was used (Santa Cruz Biotechnology, Santa Cruz, CA). Strong YY-1 binding to the consensus sequence was seen (lane 1, arrow). Supershift (lane 2, asterisk) and cold competition (lane 3) confirmed the identity and specificity of the YY1 band. When a probe for Site 1 was used, no binding or supershift was seen (lanes 4-5), indicating that it is not involved in YY1's regulation of survivin. When a probe for Site 2 was used, a double band was seen at the appropriate location, possibly representing two different binding complexes involving YY1. These bands were supershifted with the addition of YY1 antibody, and cold competition confirmed the specificity of the results. Binding was enhanced by use of a probe containing both putative YY1 binding sites (lane 10). Supershift and cold competitive again confirmed the specificity of the binding (lanes 11-12). These results indicate that the most proximal putative YY1 binding site located on the survivin promoter is a target of YY1 binding and regulation, but binding to this site is increased with the inclusion of the second YY1 recognition site.

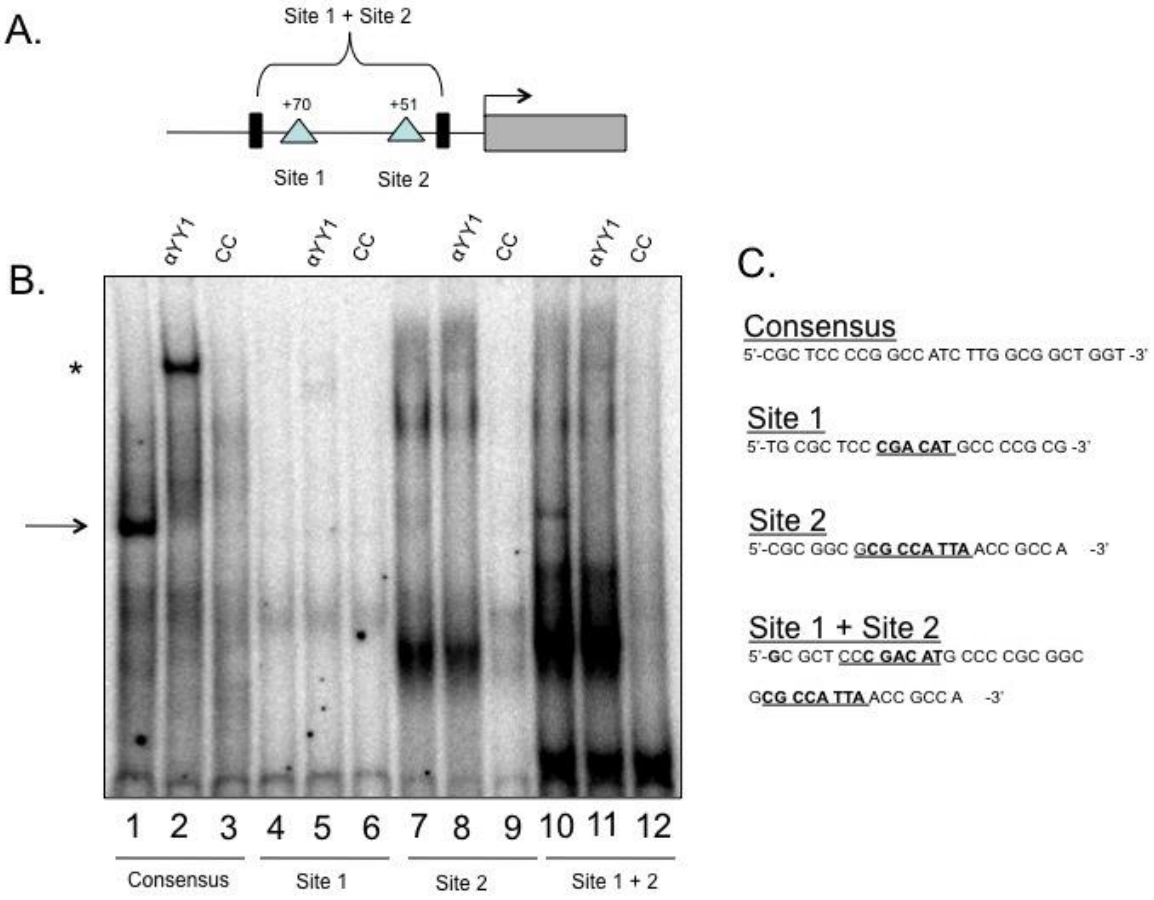


Figure 6: YY1 directly interacts with the survivin promoter. (A) Schematic of survivin promoter representing regions investigated for YY1 binding. (B) Electrophoretic mobility shift assay. Nuclear extract was prepared from U2OS cells. 32p labelled probes (C) were incubated with nuclear extracts either alone (Lanes 1,4,7, 10), with anti-YY1 antibody (lanes 2, 5, 8, 11) or cold competitor (CC) probes in 100x excess (lanes 3, 6, 9, 12). Arrow indicates YY1 bound to probe. * indicates supershift. (C) Probes used in EMSA, with underlined segments representing putative YY1 binding sites. Result is representative of two experiments showing similar results.

Discussion

YY1 is increasingly recognized as a transcription factor that plays an important role in cancer, although there is a great deal of controversy as to whether YY1 promotes or inhibits cancer development and progression. While many studies have focused on YY1 expression levels in tissue samples and the correlation of YY1 levels with clinical outcomes, metastasis free intervals, and response to therapy, far fewer studies have identified molecular mechanisms by which this multifunctional transcription factor is contributing to cancer pathology. We believe that this work shows, for the first time, a direct role for YY1 in survivin transcription, and that YY1 contributes to reduced basal expression levels of survivin. However, in the current study the osteosarcoma cell line U2OS was utilized, and it is therefore unclear if this observed transcriptional repression of survivin by YY1 is generalizable to a broad array of cancer types. Zhang et al. studied the role of YY1 on anti-apoptotic factors in colorectal carcinoma and found that siRNA-mediated knockdown of YY1 in HCT116 and LOVO cell lines resulted in a decrease in survivin protein levels and increased levels of apoptosis [106]. This is also consistent with clinical findings of a correlation between high YY1 levels and increased disease aggressiveness in various cancer types. Indeed de Nigris et al. found that in patients with osteosarcoma, YY1 overexpression correlated with increased metastasis and poor clinical outocome [107]. They also found that deletion of YY1 in the osteosarcoma cell line SaOS-2 lead to decreased cellular invasion and metastasis, possibly related to VEGF and CXCR4 regulation [108]. Other work has found that YY1 levels correlate with longer survival and decreased invasive potential. In follicular lymphoma biopsy samples, higher expression levels of YY1 correlated with longer patient survivial [82]. Wang et al. (2007) found that YY1 contributed to the increased expression of the tumor suppressor HLJ1 and related

decreased in in vitro cancer cell invasiveness. The transcriptional and post-transcriptional network regulation survivin expression is complex (reviewed by Zhang et al. [109]), and it is therefore possible that downstream of YY1's downregulation of survivin transcription other factors are significantly involved in determining the ultimate expression of survivin and the clinical sequelae that result. It is also important to note that while our work demonstrates robust surivivin promoter activity reduction via reporter assay, the resulting reduction in mRNA and protein is more modest. The extent to which YY1-mediated reduction of survivin expression results in increased apoptosis, alterations in cell cycle progression, or modulation of other hallmarks of cancer progression is currently under investigation in our laboratory.

Work by Affar et al. [63] previously showed that in an mouse YY1 knockdown model, survivin (BIRC5) levels were decreased. This lead us to initially hypothesize that YY1 overexpression in our hands would show a positive correlation with survivin expression. We observed instead a robust negative correlation between YY1 overexpression and survivin promoter activity that was also seen, although more modestly, at the protein level. Interestingly, when the human survivin core promoter sequence is compared to the mouse survivin core promoter, of note is a lack of homology at both putative YY1 sites investigated in this study with site-directed mutagenesis (Fig. 7). This may, at least in part, explain why YY1 appears to negatively regulate survivin transcription in our cell culture model, whereas in mice it may positively regulation survivin expression.

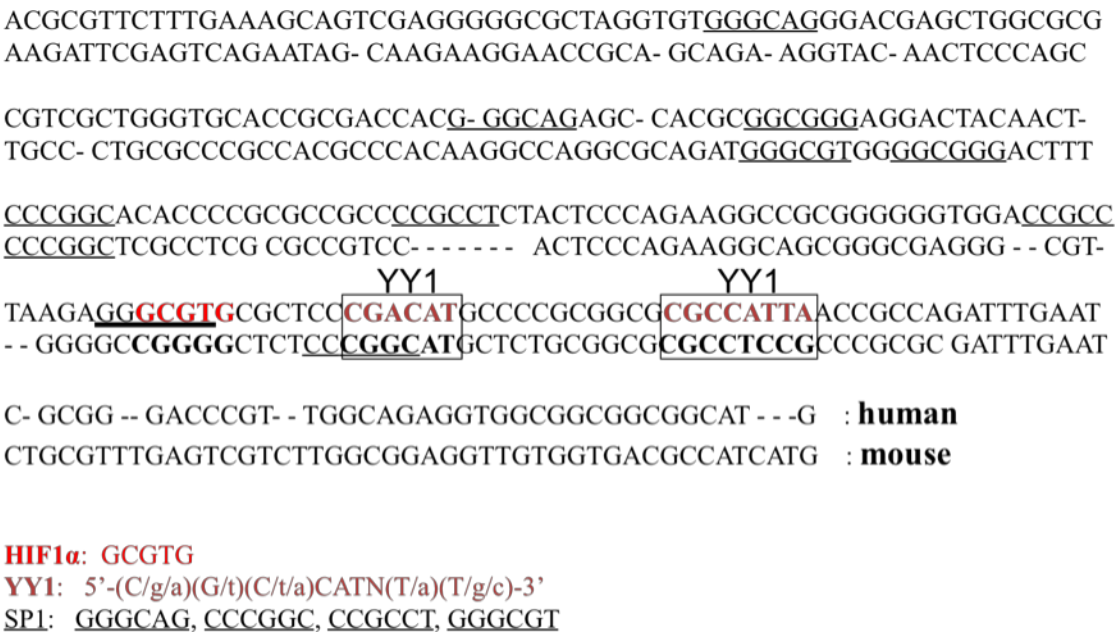


Figure 7. Comparison of human and mouse survivin promoter sequences. Boxed segments represent the 2 putative YY1 binding segments of the survivin promoter contained within the pLuc230 construct that were mutated in previous experiments. There is lack of homology between mouse and human at both putative YY1 binding sites found in the first 230 bp of the survivin promoter. HIF-1α and Sp1 bindings sites are shown for reference.

We provide several lines of evidence that YY1 represses survivin promoter activity in U2OS cells. YY1 can regulate targets genes through a host of mechanisms including protein-protein interactions that allow it to act as a coactivator or corepressor and by direct DNA binding. In the present study we identified a sequence of the survivin core promoter containing a putative YY1 binding and went on to show that YY1 is capable of binding directly to the most proximal of these putative binding sites. Binding affinity for the survivin promoter was lower than for a consensus sequence known to efficiently bind YY1 [110], and we believe that this likely represents a technical limitation owing to the extremely high GC content (70-80%) of the survivin promoter. Because of the highly complicated nature of transcriptional regulatory networks, it is also possible that a proper scaffold is required for optimal binding. Although YY1 binding occurs at the most proximal site on the survivin promoter (Site 2), binding is improved by inclusion of a second putative YY1 binding site (Site 1) that by itself does not appear to facilitate YY1 binding (see Fig. 6).

There are many proposed models for how YY1 exerts its activating or repressing effects on promoters (reviewed by Gordon et al. [70]), including displacement of transcriptional activators. Sp1, a known activator of survivin transcription, is also known to physically interact with YY1 [111,112]. Known Sp1 binding sites are located in close proximity to the YY1 binding sites examined in the current study, so it would therefore be valuable to design future studies to explore the role of Sp1 in YY1-mediates survivin repression. Also, the known repressor of survivin transcription p53 [96,113] is itself negatively regulated by YY1 [75]. Therefore future studies should also examine the interplay of p53 and YY1 expression in survivin transcriptional regulation.

It was previously believed that targeting of transcription factors as means of cancer therapy was not practical owing to the complexity of transcriptional networks. However, it is increasingly believed that drug or small molecule inhibitor-mediated interruption of transcription factor binding represents an important approach to cancer therapeutics. The small molecule inhibitor YM155 is currently in phase II clinical trials for several types of cancer including diffuse large B-cell lymphoma [46], prostate cancer [47], melanoma [48], and non-small cell lung cancer [49] due to it's previously observed ability to induced apoptosis and reduce tumor bulk in various in vitro and in vivo models [50]. Reduction in survivin transcription after YM155 treatment has been reported [114] and is believed to be a key mechanism for the apparent sensitization of tumors to cell death that has been observed.

Our discovery of a novel transcriptional repressor of survivin may provide new ways of understanding survivin expression in the context of cellular stress resulting from chemo- and radiotherapy. We also provide evidence for a possible positive role in YY1 overexpression in human cancer. The clinical significance of this finding across different cancer types has yet to be determined.

Materials and Methods

Antibodies and DNA Vectors

All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) unless otherwise indicated. The plasmid expressing YY1 protein, pcDNA3/YY1 as well as the U6/yy1 siRNA and control U6/scrambled were kind gifts of Dr. Sui, Wake Forest and were described previously [75]. Survivin nested deletion constructs were

previously described [36] and were a kind gift from Dr. Li, Roswell Park Memorial Institute.

Cell Culture and Transfection

The U2OS human osteosarcoma cell line was obtained from ATCC. U_2OS cells with stable Tet-off YY1 were a kind gift from Dr. Sui, Wake Forest and were previously described [75]. Both cell lines were maintained under an atmosphere of 5% CO_2 at 37°C in McCoys 5A media supplemented with 10% fetal bovine serum, 2 mmol/L of l-glutamine, and penicillin-streptomycin. The Tet-off cells were additionally maintained in G418, hygromycin B, and the tetracycline analogue doxycycline (50 ng/mL). YY1 expression was induced by transferring the cells to Tet-off medium, which is the same as control (Tet-on) medium except for the lack of doxycycline [75].

Transient Transfection and Reporter Assays

U2OS cells were seeded in 12-well plates and grown to 60 - 80% confluence. A total 0.4 ug of the various survivin promoter-luciferase reporter plasmids were cotransfected with either 0.6 μg of pcDNA/YY1 or empty vector expression plasmids and 0.01 ug of pRL-tk using FuGENE 6 (Roche, Indianapolis, IN). Approximately 24h after transfection, cells were lysed and assayed for luciferase activity by luminometer (Turner Design Systems, Sunnyvale, CA). Luciferase activity measurement was accomplished according to manufacturer's instructions, with the noted modifications. Briefly, cells were washed in PBS, pH 7.4, solubilized in 1 x lysis buffer (Promega, Madison, WI) and scraped with a rubber policeman, then a 5 uL aliquot of the supernatant was mixed with 20 uL of Dual-Glo® Luciferase Reagent (Promega, Madison, WI) and analysed on a Lumat

luminometer. 20 uL of Dual-Glo® Stop & Glo® Reagent was then added and a second reading on the Lumat luminometer was taken. A ratio of luminescence of the first measurement (pGL3-survivin) to the second measurement (constitutively active pRL-tk) was calculated and reported for each vector and transcription factor combination tested. The pLuc230 vector containing CAT→GGG mutation used for reporter experiments was purchased from Origene, Rockville, MD.

Western Blots

Cells were solubilized, proteins (20–40 μg) separated using 12 % Bis-Tris polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Millipore) and probed using the following antibodies: mouse monoclonal anti-YY1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and rabbit polyclonal anti-survivin (Novus, Littleton, CO). Secondary antibodies (IR-Dye-conjugated) were goat anti-rabbit and goat anti-mouse immunoglobulin (LICOR, Lincoln, Nebraska). Immunoreactive bands were detected using the Odyssey imaging system (LICOR) and quantified using ImageQuant software.

Reverse Transcriptase-PCR

Total RNA was extracted from cells at various time intervals using TRI-Reagent (Sigma, St. Louis, MO) and reverse-transcribed with SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen™, Carlsbad, CA), as described by the manufacturer and amplified with survivin-specific primers. One hundred nanograms of the resulting first-strand cDNA was used as template and amplified by PCR. Sequences of the

oligonucleotide primer sets used for reverse transcription-PCR analysis are as follows: 5′-GCA TGG CTG CCC CGA CGT TG -3′ (sense) and 5′-GCT CCG GCC AGA GGC CTC AA -3′ (antisense) for survivin, 5′-GCT TCG AGG ATC AGA TTC TCA TCC -3′ (sense) and 5′- GAC TAC ATT GAA CAA ACG CTG GTC -3′ (antisense) for YY1, 5′-GCC AGA TCT CGG CGA AGT AAA -3′ (sense) and 5′- ATA TCC AGG CTG TGT CGA CTG -3′ (antisense) for HIF1, 5′- ATG ACT CGC GAT TTC AAA CCT -3′ (sense) and 5′- CTT TGA AGT CGA GAA TCC ATT -3′ (antisense) for p75/LEDGF, and, 5′-CTCATGACCACAGTCCATGC-3′ (sense) and 5′-TTACTCCTTGGAGGCCATGT-3′ (antisense) for beta actin. Products were visualized on ethidium bromide-stained agarose gels. Amplification of beta actin served as an internal control.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared as previously described [115], with the only modification that that N-N-(L-3- trans-carboxyoxirane-2-carbonyl)-L-leucyl-agmatine (E64) and 4-(2-Aminoethyl)-benzolsulfonyl¯ourid (`Pefabloc SC') were included as protease inhibitors in concentrations suggested by the manufacturer (Boehringer, Mannheim, Germany). Protein concentration in nuclear extracts was determined using the BCA assay (Pierce) according to the manufacturers instructions. Oligos used were as follows: Two YY1 sites (YY1 sites underlined): 5'- **G**C GCT CC**C GAC ATG** CCC CGC GGC G**CG CCA TTA** ACC GCC A -3'; YY1 Site 1 5'- TG CGC TCC **CGA CAT** GCC CCG CG -3'; YY1 Site 2 CGC GGC G**CG CCA TTA** ACC GCC A -3' YY1 Consensus 5'-CGC TCC CCG GCC ATC TTG GCG GCT GGT-3'. All oligos were annealed by incubating at 95% for 2 min, then cooling to room temperature slowly. The DNA-protein binding reaction was performed in 20 ul reaction mixtures including 10% glycerol, 12 mM

HEPES ph 7.9, 4 mM TrisHCl ph 8.0, 1 mM EDTA, and 3 ug BSA. Binding reactions were incubated at room temperature for 30 minutes, then for an addition 60 minutes at 4 degrees C with anti-YY1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA sc-281) added to the appropriate reactions. The DNA-protein complexes were resolved on 5.5% non-denaturating polyacrylamidgel (29 : 1 cross-linking ratio), dried and exposed using the Storm 860 Phosphoimager (Amersham Biosciences).

Statistical Analysis

All data in reporter assay and semiquantitative PCR are presented as means ± standard deviation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

NRG carried out most of the experiments and contributed to the writing of the manuscript. JMS performed all of the flow cytometry experiments, FL and GS contributed reagents such as cell lines and plasmids and contributed to data interpretation and critical revision of the manuscript. NRW contributed to the conception and design of the entire study, wrote, with NRG, the manuscript and conducted the final editing of the manuscript.

Acknowledgments

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References

1. Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 2003;22:8581-9.

2. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580-4.

3. Altieri DC. The case for survivin as a regulator of microtubule dynamics and cell-death decisions. Curr Opin Cell Biol 2006;18:609-15.

4. Li F. Survivin study: what is the next wave? J Cell Physiol 2003;197:8-29.

5. Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC. Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. Am J Pathol 1998;152:43-9.

6. Fukuda S, Pelus LM. Survivin, a cancer target with an emerging role in normal adult tissues. Mol Cancer Ther 2006;5:1087-98.

7. Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, thor Straten P. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. Cancer Res 2001;61:5964-8.

8. Grossman D, Kim PJ, Schechner JS, Altieri DC. Inhibition of melanoma tumor growth in vivo by survivin targeting. Proc Natl Acad Sci U S A 2001;98:635-40.

9. Mesri M, Wall NR, Li J, Kim RW, Altieri DC. Cancer gene therapy using a survivin mutant adenovirus. J Clin Invest 2001;108:981-90.

10. Wall NR, O'Connor DS, Plescia J, Pommier Y, Altieri DC. Suppression of survivin phosphorylation on Thr34 by flavopiridol enhances tumor cell apoptosis. Cancer Res 2003;63:230-5.

11. Plescia J, Salz W, Xia F, et al. Rational design of shepherdin, a novel anticancer agent. Cancer Cell 2005;7:457-68.

12. Olie RA, Simoes-Wust AP, Baumann B, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000;60:2805-9.

13. Kanwar JR, Shen WP, Kanwar RK, Berg RW, Krissansen GW. Effects of survivin antagonists on growth of established tumors and B7-1 immunogene therapy. J Natl Cancer Inst 2001;93:1541-52.

14. Andersen MH, Svane IM, Becker JC, Straten PT. The universal character of the tumor-associated antigen survivin. Clin Cancer Res 2007;13:5991-4.

15. Ling X, Li F. Silencing of antiapoptotic survivin gene by multiple approaches of RNA interference technology. Biotechniques 2004;36:450-4, 6-60.

16. Tu SP, Jiang XH, Lin MC, et al. Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer. Cancer Res 2003;63:7724-32.

17. Pennati M, Binda M, Colella G, et al. Ribozyme-mediated inhibition of survivin expression increases spontaneous and drug-induced apoptosis and decreases the tumorigenic potential of human prostate cancer cells. Oncogene 2004;23:386-94.

18. Shen C, Buck A, Polat B, et al. Triplex-forming oligodeoxynucleotides targeting survivin inhibit proliferation and induce apoptosis of human lung carcinoma cells. Cancer Gene Ther 2003;10:403-10.

19. Raj D, Liu T, Samadashwily G, Li F, Grossman D. Survivin repression by p53, Rb and E2F2 in normal human melanocytes. Carcinogenesis 2008;29:194-201.

20. Gritsko T, Williams A, Turkson J, et al. Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. Clin Cancer Res 2006;12:11-9.

21. Peng XH, Karna P, Cao Z, Jiang BH, Zhou M, Yang L. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. J Biol Chem 2006;281:25903-14.

22. Dasgupta P, Kinkade R, Joshi B, Decook C, Haura E, Chellappan S. Nicotine inhibits apoptosis induced by chemotherapeutic drugs by up-regulating XIAP and survivin. Proc Natl Acad Sci U S A 2006;103:6332-7.

23. Li Y, Xie M, Yang J, et al. The expression of antiapoptotic protein survivin is transcriptionally upregulated by DEC1 primarily through multiple sp1 binding sites in the proximal promoter. Oncogene 2006;25:3296-306.

24. Li F, Altieri DC. Transcriptional analysis of human survivin gene expression. Biochem J 1999;344 Pt 2:305-11.

25. Cosgrave N, Hill AD, Young LS. Growth factor-dependent regulation of survivin by c-myc in human breast cancer. Journal of molecular endocrinology 2006;37:377-90.

26. Zhu N, Gu L, Findley HW, et al. KLF5 Interacts with p53 in regulating survivin expression in acute lymphoblastic leukemia. J Biol Chem 2006;281:14711-8.

27. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 2002;277:3247-57.

28. Xu Y, Fang F, Ludewig G, Jones G, Jones D. A mutation found in the promoter region of the human survivin gene is correlated to overexpression of survivin in cancer cells. DNA Cell Biol 2004;23:419-29.

29. Shi Y, Lee JS, Galvin KM. Everything you have ever wanted to know about Yin Yang 1. Biochim Biophys Acta 1997;1332:F49-66.

30. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. Oncogene 2006;25:1125-42.

31. Deng Z, Wan M, Cao P, Rao A, Cramer SD, Sui G. Yin Yang 1 regulates the transcriptional activity of androgen receptor. Oncogene 2009.

32. Wang X, Feng Y, Xu L, et al. YY1 restrained cell senescence through repressing the transcription of p16. Biochim Biophys Acta 2008;1783:1876-83.

33. Harrison L, Blackwell K. Hypoxia and anemia: factors in decreased sensitivity to radiation therapy and chemotherapy? Oncologist 2004;9 Suppl 5:31-40.

34. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996;56:4509-15.

35. Bottaro DP, Liotta LA. Cancer: Out of air is not out of action. Nature 2003;423:593-5.

36. Chang Q, Qin R, Huang T, Gao J, Feng Y. Effect of antisense hypoxia-inducible factor 1alpha on progression, metastasis, and chemosensitivity of pancreatic cancer. Pancreas 2006;32:297-305.

37. Mediavilla-Varela M, Pacheco FJ, Almaguel F, et al. Docetaxel-induced prostate cancer cell death involves concomitant activation of caspase and lysosomal pathways and is attenuated by LEDGF/p75. Mol Cancer 2009;8:68.

38. Fatma N, Singh DP, Shinohara T, Chylack LT, Jr. Transcriptional regulation of the antioxidant protein 2 gene, a thiol-specific antioxidant, by lens epithelium-derived growth factor to protect cells from oxidative stress. J Biol Chem 2001;276:48899-907.

39. Wei H, Wang C, Chen L. Proliferating cell nuclear antigen, survivin, and CD34 expressions in pancreatic cancer and their correlation with hypoxia-inducible factor 1alpha. Pancreas 2006;32:159-63.

40. Bai H, Ge S, Lu J, Qian G, Xu R. Hypoxia inducible factor-1alpha-mediated activation of survivin in cervical cancer cells. The journal of obstetrics and gynaecology research 2013;39:555-63.

41. Hyde-DeRuyscher RP, Jennings E, Shenk T. DNA binding sites for the transcriptional activator/repressor YY1. Nucleic Acids Res 1995;23:4457-65.

42. Singh DP, Fatma N, Kimura A, Chylack LT, Jr., Shinohara T. LEDGF binds to heat shock and stress-related element to activate the expression of stress-related genes. Biochem Biophys Res Commun 2001;283:943-55.

43. Li F, Altieri DC. The cancer antiapoptosis mouse survivin gene: characterization of locus and transcriptional requirements of basal and cell cycle-dependent expression. Cancer Res 1999;59:3143-51.

44. Sui G, Affar el B, Shi Y, et al. Yin Yang 1 is a negative regulator of p53. Cell 2004;117:859-72.

45. Zhang N, Li X, Wu CW, et al. microRNA-7 is a novel inhibitor of YY1 contributing to colorectal tumorigenesis. Oncogene 2012.

46. de Nigris F, Zanella L, Cacciatore F, et al. YY1 overexpression is associated with poor prognosis and metastasis-free survival in patients suffering osteosarcoma. BMC Cancer 2011;11:472.

47. de Nigris F, Crudele V, Giovane A, et al. CXCR4/YY1 inhibition impairs VEGF network and angiogenesis during malignancy. Proc Natl Acad Sci U S A 2010;107:14484-9.

48. Naidoo K, Clay V, Hoyland JA, et al. YY1 expression predicts favourable outcome in follicular lymphoma. J Clin Pathol 2011;64:125-9.

49. Zhang M, Yang J, Li F. Transcriptional and post-transcriptional controls of survivin in cancer cells: novel approaches for cancer treatment. J Exp Clin Cancer Res 2006;25:391-402.

50. Affar EB, Gay F, Shi Y, et al. Essential Dosage-Dependent Functions of the Transcription Factor Yin Yang 1 in Late Embryonic Development and Cell Cycle Progression. Mol Cell Biol 2006;26:3565-81.

51. Ai W, Liu Y, Wang TC. Yin yang 1 (YY1) represses histidine decarboxylase gene expression with SREBP-1a in part through an upstream Sp1 site. Am J Physiol Gastrointest Liver Physiol 2006;290:G1096-104.

52. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. Oncogene 2005;25:1125-42.

53. Lee JS, Galvin KM, Shi Y. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. Proc Natl Acad Sci U S A 1993;90:6145-9.

54. Seto E, Lewis B, Shenk T. Interaction between transcription factors Sp1 and YY1. Nature 1993;365:462-4.

55. Mirza A, McGuirk M, Hockenberry TN, et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002;21:2613-22.

56. Cheson BD, Bartlett NL, Vose JM, et al. A phase II study of the survivin suppressant YM155 in patients with refractory diffuse large B-cell lymphoma. Cancer 2012;118:3128-34.

57. Tolcher AW, Quinn DI, Ferrari A, et al. A phase II study of YM155, a novel small-molecule suppressor of survivin, in castration-resistant taxane-pretreated prostate cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 2012;23:968-73.

58. Lewis KD, Samlowski W, Ward J, et al. A multi-center phase II evaluation of the small molecule survivin suppressor YM155 in patients with unresectable stage III or IV melanoma. Investigational new drugs 2011;29:161-6.

59. Giaccone G, Zatloukal P, Roubec J, et al. Multicenter phase II trial of YM155, a small-molecule suppressor of survivin, in patients with advanced, refractory, non-small-cell lung cancer. J Clin Oncol 2009;27:4481-6.

60. Nakahara T, Kita A, Yamanaka K, et al. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. Cancer Res 2007;67:8014-21.

61. Tao YF, Lu J, Du XJ, et al. Survivin selective inhibitor YM155 induce apoptosis in SK-NEP-1 Wilms tumor cells. BMC Cancer 2012;12:619.

62. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.

CHAPTER THREE

ENHANCED ANTITUMOR EFFECT OF COMBINED GEMCITABINE AND PROTON RADIATION IN THE TREATMENT OF PANCREATIC CANCER

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Key Words: Gemcitabine; Proton Irradiation; short inhibitory RNA; Survivin; XIAP; polyploidy; Inhibitor of Apoptosis

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Abstract

OBJECTIVES: This study evaluates the efficacy of combining proton irradiation with gemcitabine and the role the inhibitor of apoptosis proteins (IAP) survivin & XIAP play in the radiosensitive vs. radioresistant status of pancreatic cancer. METHODS: The radioresistant (PANC-1) and radiosensitive (MIA PaCa-2) pancreatic carcinoma cells response to combined gemcitabine and proton irradiation was compared. Cells were treated with 0.1 - 500 μM gemcitabine and 0 - 15 Gy proton irradiation after which Trypan blue and flow cytometry were utilized to determine changes in the cell cycle and apoptosis. Expression levels of survivin were measured using Western blotting. Combination therapy with 24 h gemcitabine followed by 10-Gy proton irradiation proved most effective. RESULTS: Gemcitabine and proton irradiation, resulted in increased survivin levels, with little apoptosis. However, combination therapy resulted in robust apoptotic induction with a concomitant survivin & XIAP reduction in the MIA PaCa-2 cells with little effect in the PANC-1 cells. siRNA studies confirmed a role for XIAP in the radioresistance of PANC-1 cells. CONCLUSIONS: Our data demonstrate that combining gemcitabine and proton irradiation enhances apoptosis in human pancreatic cancer cells when XIAP levels decrease. Therefore, XIAP may play an important role in human pancreatic cancer proton radioresistance.

Introduction

Pancreatic cancer is the fourth most common cause of cancer death in men and women in the United States, with 5-year survival for all stages of disease less than 5% [116]. Pancreatic cancer has no clear early warning signs or symptoms and is usually silent until

the disease is well advanced. Patients have a median survival of 4-8 months after diagnosis due in part to the advanced stage the disease has already attained by the time it is discovered and treatment has begun. Risk factors include age with diagnosis occurring in people ages 65-79, smoking, sex, and possibly diets high in fat [117]. Currently, if diagnosed early, surgical resection remains the only viable cure. However, only 20% of pancreatic cancer patients meet these criteria [118]. It is therefore necessary to discover new therapies or therapeutic combinations in order to significantly impact this deadly disease. The anti-metabolite agent gemcitabine is currently being employed to treat pancreatic cancer [119]. While gemcitabine has shown significant benefit in clinical applications, its ability to more than modestly impact pancreatic cancer is limited. It has been speculated that combinatory treatments using gemcitabine and other chemotherapeutics or radiotherapeutics could improve survival rates [120,121]. Proton radiotherapy has been investigated for a number of cancer types including cancers of the prostate, head & neck and brain [122-124]. Protocols are also currently in progress or development for treating a variety of additional cancer types including: carcinoma of the nasopharynx, paranasal sinus carcinoma, non-small-cell lung carcinoma, hepatocellular carcinoma and pancreatic cancer [125]. Pancreatic cancers though inherently resistant to photon radiation may be safely treated using protons. Proton therapy allows dose escalation to improve local tumor control in anatomic sites and histologies where local control is suboptimal with photons [124]. This improved dose localization reduces normal-tissue doses resulting in lower acute and late toxicity.

Survivin, a member of the inhibitor of apoptosis protein (IAP) family has previously been shown to be a prognostic marker for pancreatic cancer patients [84,126,127]

and has also been implicated in cancer cell radio- and chemotherapy resistance [88]. Many recent reports have demonstrated that inhibiting survivin expression by antisense oligonucleotides [128], dominant negative mutation [14,129], and ribozyme [130] can reduce cancer cell radio- and chemoresistance and may be important to resensitize these tumors to therapy.

The goal of this study was to examine the combined affect of gemcitabine and proton irradiation on the pancreatic cell lines PANC-1 (photon radioresistant) and MIA PaCa-2 (photon radiosensitive) and to determine whether the same survivin involvement in proton radiation resistance would be observed[129,131,132].

Materials and Methods

Cell Cultures

Pancreatic carcinoma (Panc-1 & MIA Paca-2) cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 100 units of penicillin, 100 μg/ml streptomycin, 300 μg of L-glutamine and 10% heat inactivated FBS (ATCC). MIA PaCa-2 media also included 2.5% horse serum (ATCC). Cells were grown at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. Gemcitabine or Gemzar™ (Eli Lilly and Company, Indianapolis, Indiana) was dissolved in water and added to cells for the duration of 24 hours prior, simultaneously or 24 hours after radiation exposure. Post treatment, the cells were returned to the incubator for an additional 24, 48, or 72 h. All radiation procedures were accomplished in the Loma Linda University Radiobiology Proton Treatment Facility, now the James M. Slater, MD, Proton Treatment and Research Center. Cells were exposed in vitro to 250 MeV

protons with doses ranging from 0 to 15 Gy at four different dose rates: a low dose rate of 2.5 Gy/h, an intermediate dose rate of 5 Gy/h and two high dose rates of 10 and 15 Gy/h. Cells are treated as shown in Figure 1.

Apoptosis and Cell Cycle Analysis

Subconfluent cultures of the various cell lines were incubated with vehicle (water), gemcitabine (0 to 500 μM) or exposed to proton irradiation (0 to 15 Gy/h) for 0, 24, 48, and 72 hours at 37°C or combinations of gemcitabine and proton irradiation described above. Cells were harvested, prepared, and analyzed for DNA content as described previously [133]. DNA content was analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA). The distribution of cells in the different phases of the cell cycle was analyzed from DNA histograms using BD CellQuest software (Becton Dickinson and Company, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR).

Western Blot Analysis

Cells were solubilized, proteins (20–40 μg) separated using 12 or 15% Bis-Tris polyacrylamide gels, proteins transferred onto nitrocellulose membranes (Bio-Rad) and probed using the following antibodies: rabbit polyclonal anti-survivin (Novus, Littleton, CO) and GAPDH (Cell Signaling Technologies, Beverly, MA), and polyclonal β-actin (Abcam, Cambridge, MA). Secondary antibodies (IR-Dye-conjugated) were goat anti-rabbit immunoglobulin (LICOR, Lincoln, Nebraska). Immunoreactive bands were detected using the Odyssey imaging system (LICOR) and quantified using ImageQuant

software. Protein quantifications presented in this report were normalized with respect to GAPDH or β-actin as indicated.

siRNA Knockdown

siRNA oligos were obtained for Survivin and XIAP knockdown (Santa Cruz Biotechnology, Santa Cruz, CA). In addition, a scramble siRNA was purchased for control. Amaxa Nucleofection technology was employed for transfection of PANC-1 cells. PANC-1 cells were cultured as described above and passaged 3 days before transfection. Nucleofection Kit R was used. PANC-1 cells were trypsinized, counted, and aliquoted into $1x10_6$ cells per tube. Cells were spun down and resuspended in 100 uL of nucleofection solution. To this 1.5 ug of siRNA was added, the suspension was transferred to a nucleofection cuvette, and the suggested program was applied.. Immediately after program completion, 500 uL of fresh media was added and the cells were aliquoted equally into 6-well plates for further culture and treatment. Cells were cultured for 3 days after transfection to allow for gene knockdown. After this time, the appropriate treatments were applied.

Statistical Analysis

Statistical analysis was performed using a two-way analysis of variance (ANOVA) with the aid of JMP statistical software (Cary, NC). A paired t test was used for group analysis.

Results

Gemcitabine-Induced Survivin Protein is Associated with Growth Inhibition and Cytotoxicity in Pancreatic Cancer Cells

Treatment of PANC-1 or MIA PaCa-2 cells with various concentrations (100 μM, 10 μM and 1.0 μM) of gemcitabine resulted in a modest G_0/G_1 phase cell cycle arrest at 24 h, followed by the progressive appearance of apoptosis over the 48-72 h time interval (Figure 2A). Dose escalation of gemcitabine was insignificant, as 1 μM was as effective as 100 μM in inducing cell cycle arrest as well as apoptotic cell death in both cell lines. Between cell lines, the more radiosensitive MIA PaCa-2 cells were also more sensitive to gemcitabine than the radioresistant PANC-1 cells. Both cell lines in their non-treated resting state exhibited between a 10 and 20%

polyploid fraction (cells containing greater then 4N DNA). Interestingly, after cells were gemcitabine treated, this polyploid fraction disappeared in both cell lines (Figure 2A).

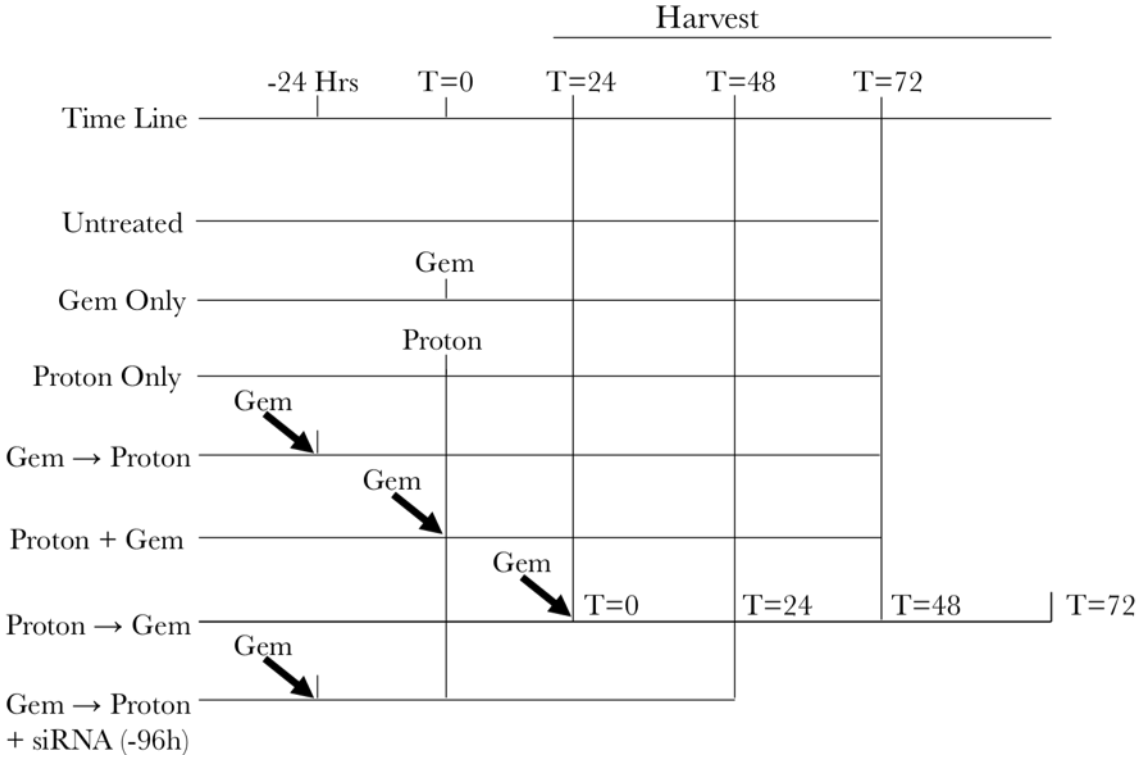

Figure 1. Treatment schematic. Gemcitabine and protons were given at time = 0. Combination treatment of gemcitabine followed by proton radiation was treated with gemcitabine given at -24 hrs and then followed by proton irradiation at time = 0 (Gem → Proton). Simultaneous treatment was accomplished with both modalities being given at time = 0 (Proton + Gem). Proton irradiation was administered 24 hrs before gemcitabine treatment at time = 24 (Proton → Gem). All cells were harvested 24, 48, and 72 hrs after its final treatment was administered.

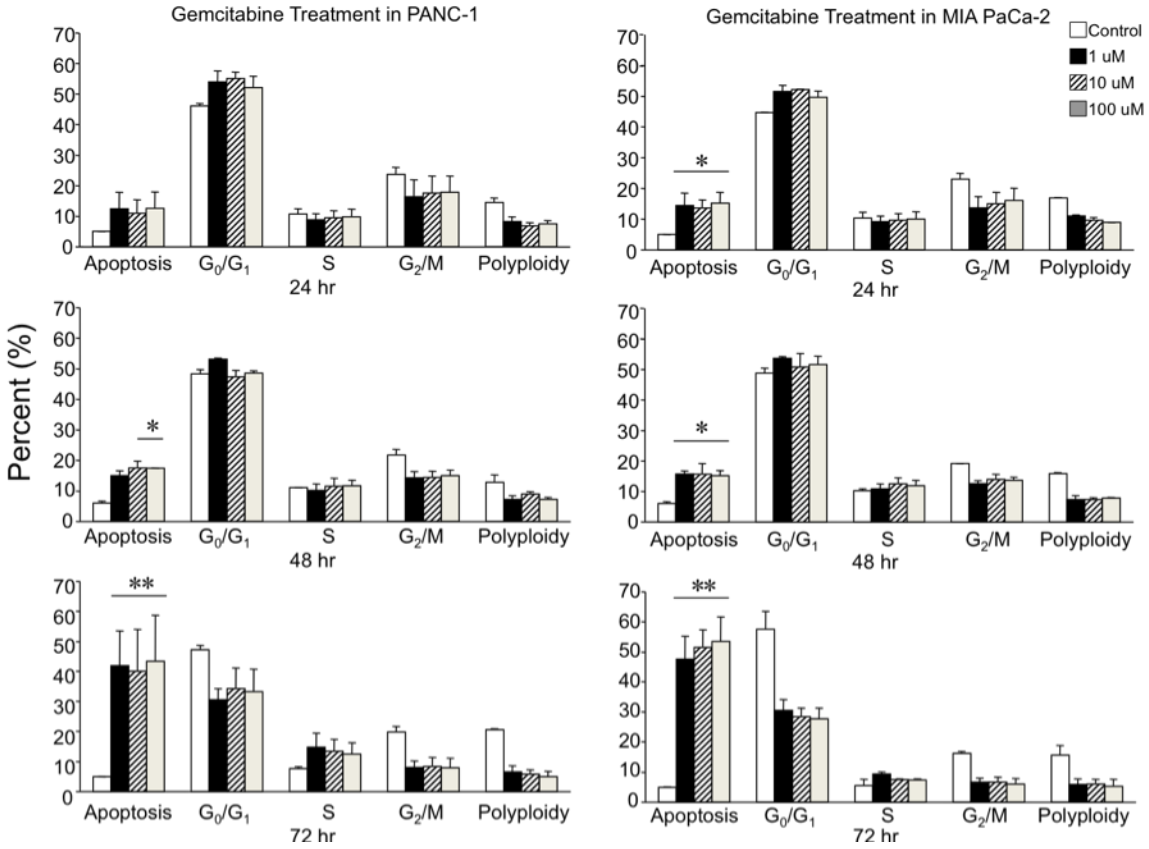

Figure 2A. Gemcitabine treatment of PANC-1 and MIA PaCa-2 cell lines. Cells were treated using 0 μM,1 μM, 10 μM, and 100 μM gemcitabine after which they were harvested and analyzed for DNA content by propidium iodide staining and flow cytometry at 24 hr, 48 hr, and 72 hr. Percentages of apoptotic cells with hypodiploid (sub-G1) DNA content as well as those in G_0/G_1, S, G_2/M and polyploid are indicated per each condition tested. Data are the mean ± SD of three independent experiments (*$p<0.01$, **$p<0.001$).

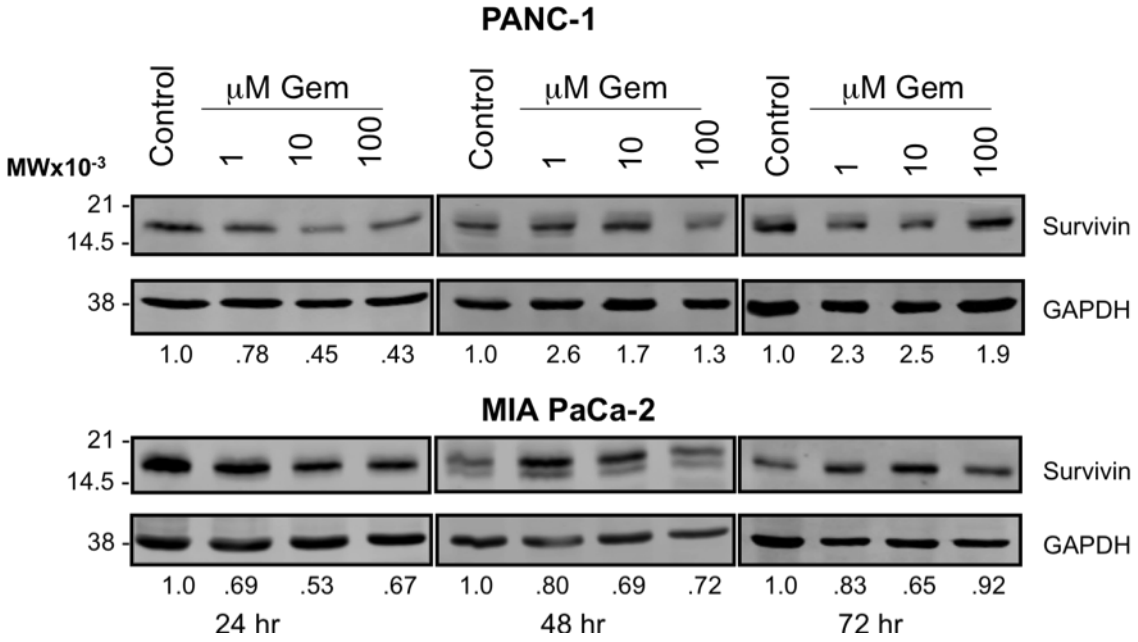

Figure 2B. Detergent-solubilized extracts of pancreatic cells treated with gemcitabine were analyzed at the indicated time intervals for reactivity with antibodies for survivin and GAPDH (loading control), by Western blotting. Molecular-weight (M_r) markers in kilodaltons are shown on the left.

Treatment of both PANC-1 and MIA PaCa-2 cell lines for 24 h with gemcitabine resulted in a dose-dependent reduction in survivin levels by Western blot analysis (Figure 2B). Further gemcitabine incubation of 48 h and 72 h resulted in survivin protein levels being enhanced or unchanged at doses of 1 and 10 μM in both cell lines, a result that is most likely due to drug-induced stress [134]. As a dose of 10 μM gemcitabine induced a time dependent G_0/G_1 arrest, enhanced cytotoxicity and 24 h reduction in survivin, this dose was chosen for all further experiments with MIA PaCa-2 cells. However, PANC-1 cells were treated with 100 μM gemcitabine due to their resistance to gemcitabine-induced cell death.

Treatment of PANC-1 or MIA PaCa-2 cells with various concentrations (0, 2.5, 5, 10, and 15 Gy) of proton irradiation resulted in significant cell cycle arrest in both the radiosensitive MIA PaCa-2 as well as the radioresistant PANC-1 pancreatic cell lines in a dose-dependent manner (Figure 3A). Unlike the results of gemcitabine in these two cell lines, only the radiation sensitive MIA PaCa-2 cells were induced to undergo notable levels of apoptosis. MIA PaCa-2 cells experienced a time and dose-dependent apoptosis with the G_2/M arrested cells being the most sensitive as it is from this population of cells that the highest level of time-dependent death is recorded. Photon radioresistant PANC-1 cells were also resistant to proton radiation (Figure 3A) even though these cells also experienced a dose-dependent cell cycle arrest. In both cell lines, proton radiation induced a dose-dependent increase in polyploid cells from the 10% observed in the untreated controls to almost 30% in those treated with 15 Gy (Figure 3A).

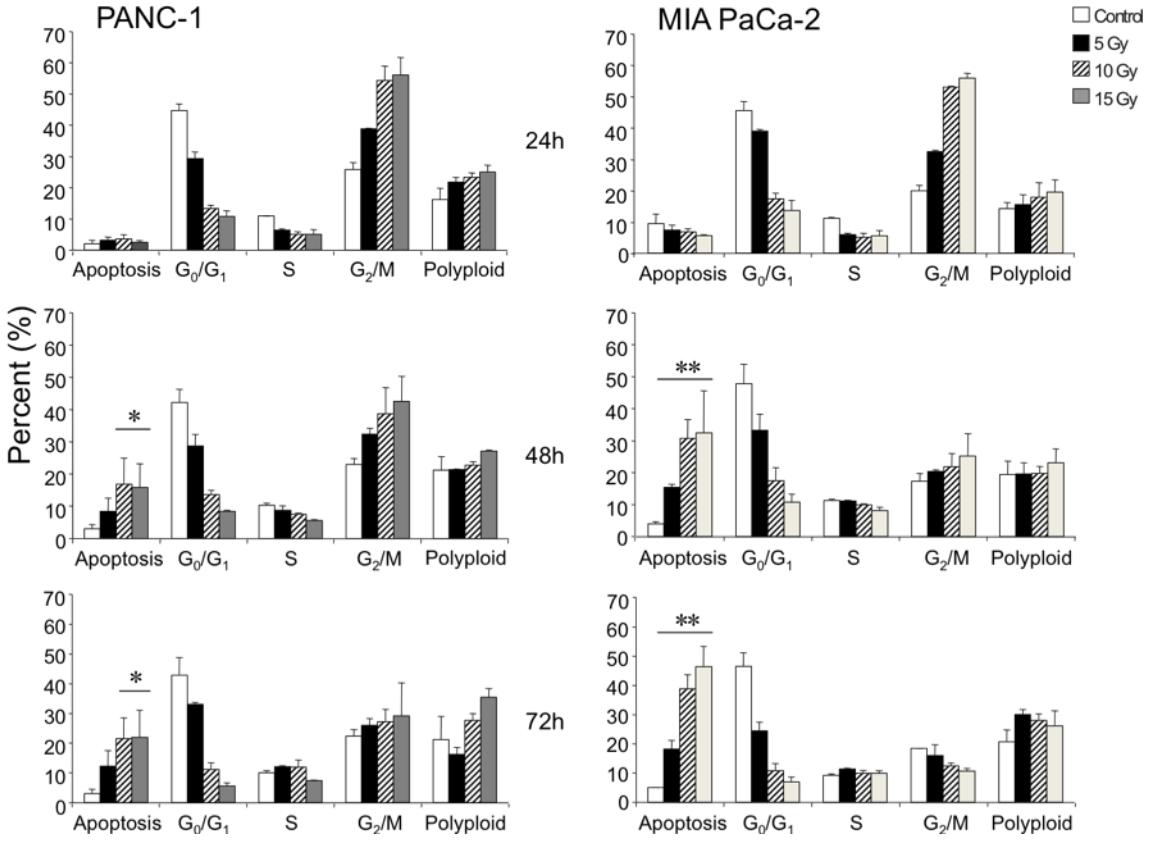

Figure 3A. Proton irradiation of PANC-1 and MIA PaCa-2 cell lines. Cells were treated using 0, 5, 10 or 15 Gy of proton radiation after which they were harvested and analyzed for DNA content by propidium iodide staining and flow cytometry at 24 hr, 48 hr, and 72 hr. Percentages of apoptotic cells with hypodiploid (sub-G1) DNA content as well as those in G_0/G_1, S, G_2/M and polyploid are indicated per each condition tested. Data are the mean ± SD of three independent experiments (*$p<0.01$, **$p<0.001$).

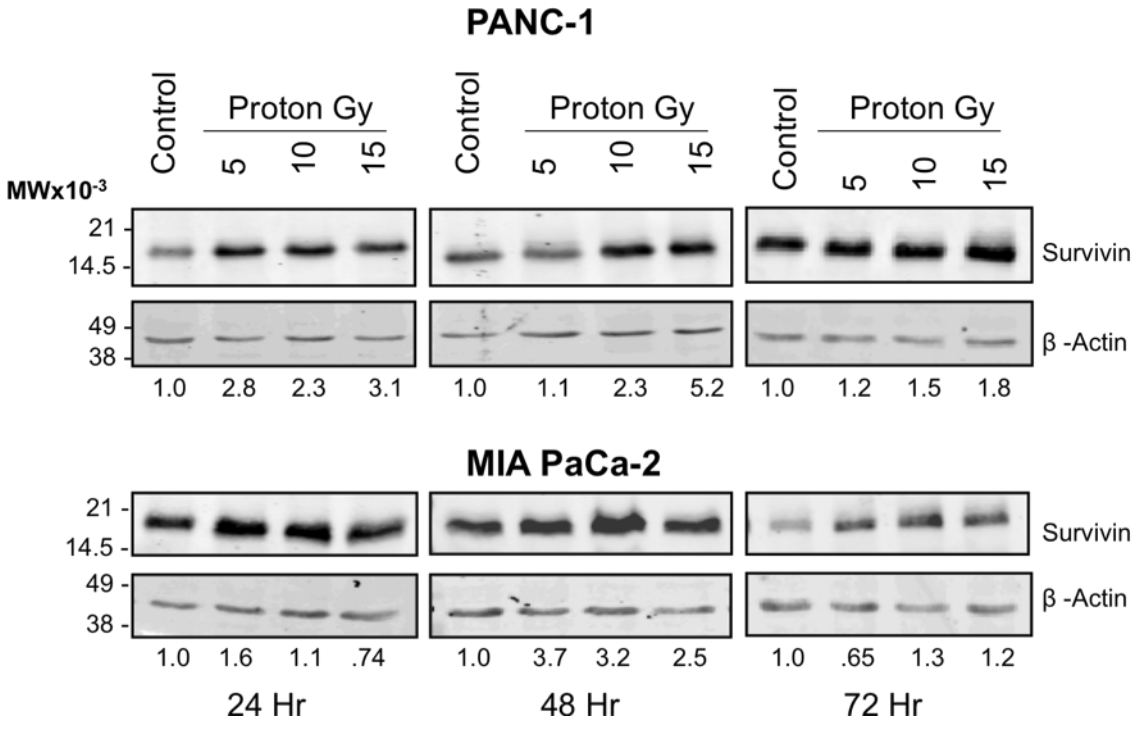

Figure 3B. Detergent-solubilized extracts of pancreatic cells treated with proton radiation were analyzed at the indicated time intervals for reactivity with antibodies for survivin and β-actin (loading control), by Western blotting. Molecular-weight (M_r) markers in kilodaltons are shown on the left.

Treatment of both MIA PaCa-2 and PANC-1 cells lines with proton radiation resulted in a dose-dependent increase in survivin protein as defined by Western blot analysis (Figure 3B). This increase in survivin protein concomitant with the observed G_2/M arrest is expected as survivin has been previously shown to be expressed during cell division in a cell cycle-dependent manner [134].

Sequential Treatment of Pancreatic Cancer Cells with Gemcitabine and Proton Irradiation Enhances the Effect of Single Agent Treatment in only MIA PaCa-2 Cells

Treatment of MIA PaCa-2 cells with 10 μM gemcitabine (Figure 2A) and 10 Gy proton radiation (Figure 3A) resulted in modest levels of apoptosis, cell cycle arrest and survivin modulation in both cell lines with the most prominent killing effect in MIA PaCa-2 cells. We therefore combined the two modalities as shown in Figure 1.

Treatment of MIA PaCa-2 cells (Figure 4A) with 10 μM gemcitabine or 10 Gy proton irradiation resulted in cell cycle arrest at G_0/G_1 and G_2/M respectively. For sequential treatments that include gemcitabine as the first modality in the treatment regimen, G_0/G_1 arrest was also the prominent phenotypic cell cycle change and likewise a G_2/M arrest resulted from sequential treatments that used proton irradiation as the first modality in the treatment regime. Cell cycle arrest was followed by the progressive appearance of apoptosis over the 72 h time interval. However, sequential treatments where gemcitabine lead proton irradiation resulted in an enhanced apoptosis by 48 h, a trend that increased further by 72 h. An interesting observation first made with the single agent treatment experiments (Figure 2 & 3) is that gemcitabine treatment does not result in significant numbers of cells having greater than 4N DNA (polyploidy) while proton

irradiation results in a progressive accumulation of polyploid cells. This is also observed in the sequential treatments where proton irradiation leads gemcitabine treatment. However, where gemcitabine and proton are given concurrently, little enhancement of this polyploid fraction is recorded and where gemcitabine leads the proton irradiation, an unremarkable number of polyploid cells are recorded (Figure 4A).

Like MIA PaCa-2 cells, treatment of PANC-1 cells (Figure 4B) with 100 μM gemcitabine or 10 Gy proton irradiation alone or those combinations that lead with gemcitabine or proton irradiation also resulted in cell cycle arrest in G_0/G_1 and G_2/M respectively. However, unlike MIA PaCa-2 cells, sequential treatments did not result in the progressive appearance of apoptotic cells, even though 10 fold higher concentration of gemcitabine was used. In fact, after 72 h of treatment, no significant changes are observed from those recorded after only 24 h of treatment. Polyploidy does however, match what was observed in the MIA PaCa-2 cells (Figure 4A).

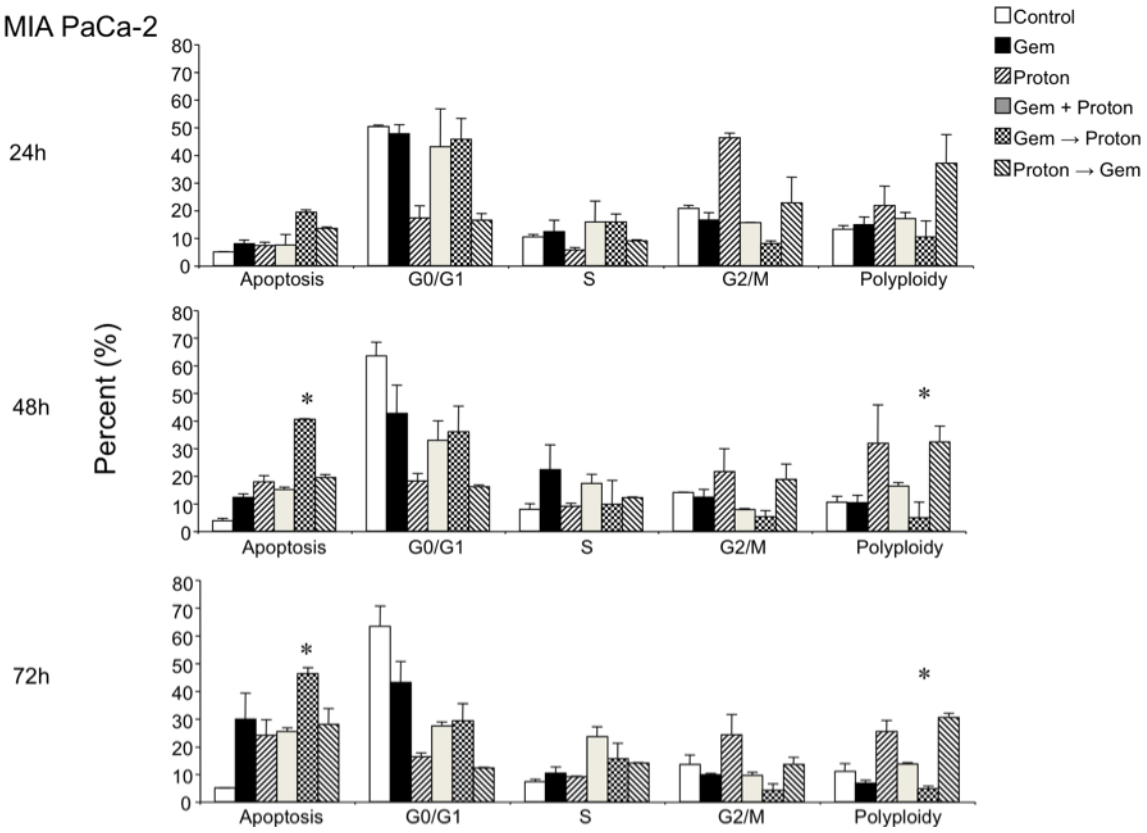

Figure 4A. Combination Gemcitabine and Proton Radiation in PANC-1 and MIA PaCa-2 cell lines. **(A)** PANC-1 and **(B)** MIA PaCa-2 cells were treated using 10 Gy of proton radiation and 10 μM gemcitabine after which they were harvested and analyzed for DNA content by propidium iodide staining and flow cytometry at 24 hr, 48 hr, and 72 hr. Percentages of apoptotic cells with hypodiploid (sub-G1) DNA content as well as those in G_0/G_1, S, G_2/M and polyploid are indicated per each condition tested. Data are the mean ± SD of three independent experiments (*$p<0.01$).

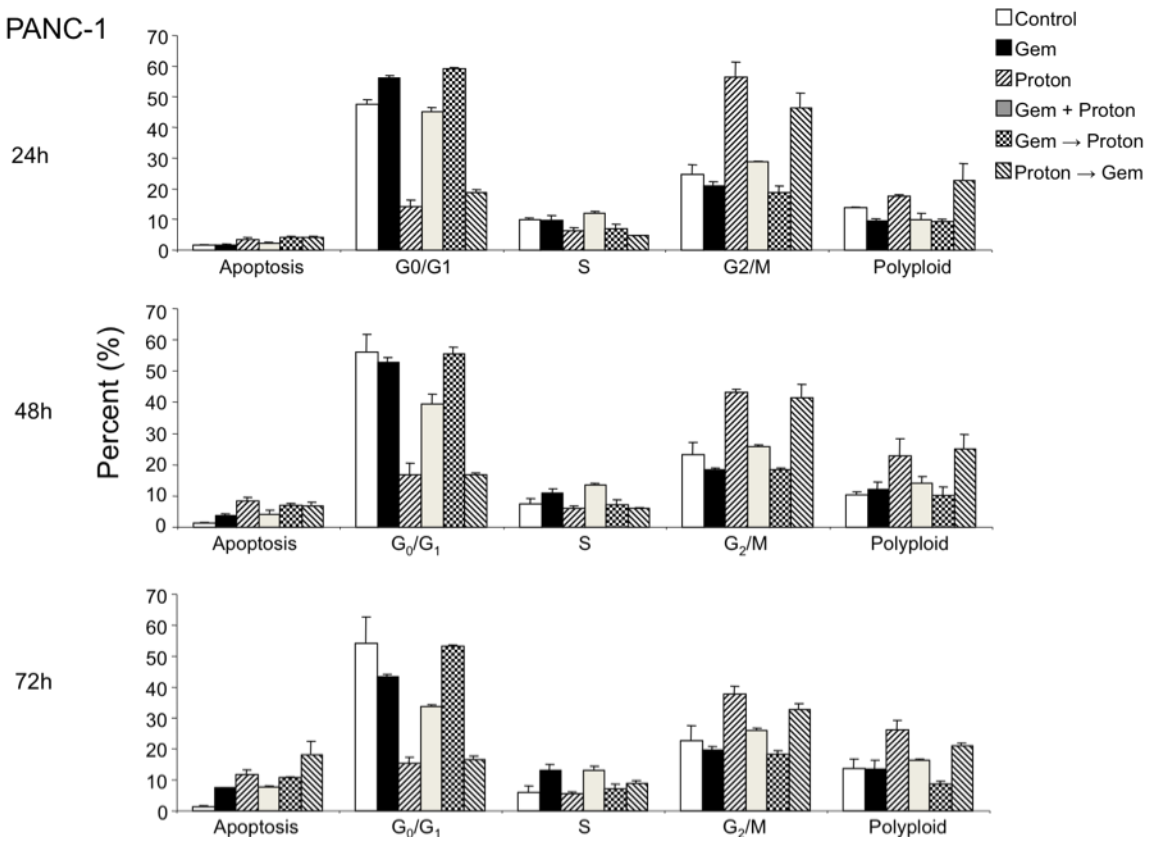

Figure 4B. Combination Gemcitabine and Proton Radiation in PANC-1 and MIA PaCa-2 cell lines. **(A)** PANC-1 and **(B)** MIA PaCa-2 cells were treated using 10 Gy of proton radiation and 10 μM gemcitabine after which they were harvested and analyzed for DNA content by propidium iodide staining and flow cytometry at 24 hr, 48 hr, and 72 hr. Percentages of apoptotic cells with hypodiploid (sub-G1) DNA content as well as those in G_0/G_1, S, G_2/M and polyploid are indicated per each condition tested. Data are the mean ± SD of three independent experiments (*$p<0.01$).

Modulation of Survivin Protein Expression by Combining Gemcitabine and Proton Irradiation in Pancreatic Cancer Cell Lines

To determine the potential relevance of targeting survivin for tumor cell apoptosis in sequential gemcitabine and proton irradiation treatments, survivin levels were analyzed by Western blotting in PANC-1 and MIA PaCa-2 cells treated with gemcitabine or proton irradiation alone or with the sequential combinations described previously at 48 h post treatment (Figure 1). Treatment of PANC-1 cells with gemcitabine or protons resulted in a 2 and 4 fold increase in survivin expression respectively (Figure 4C).

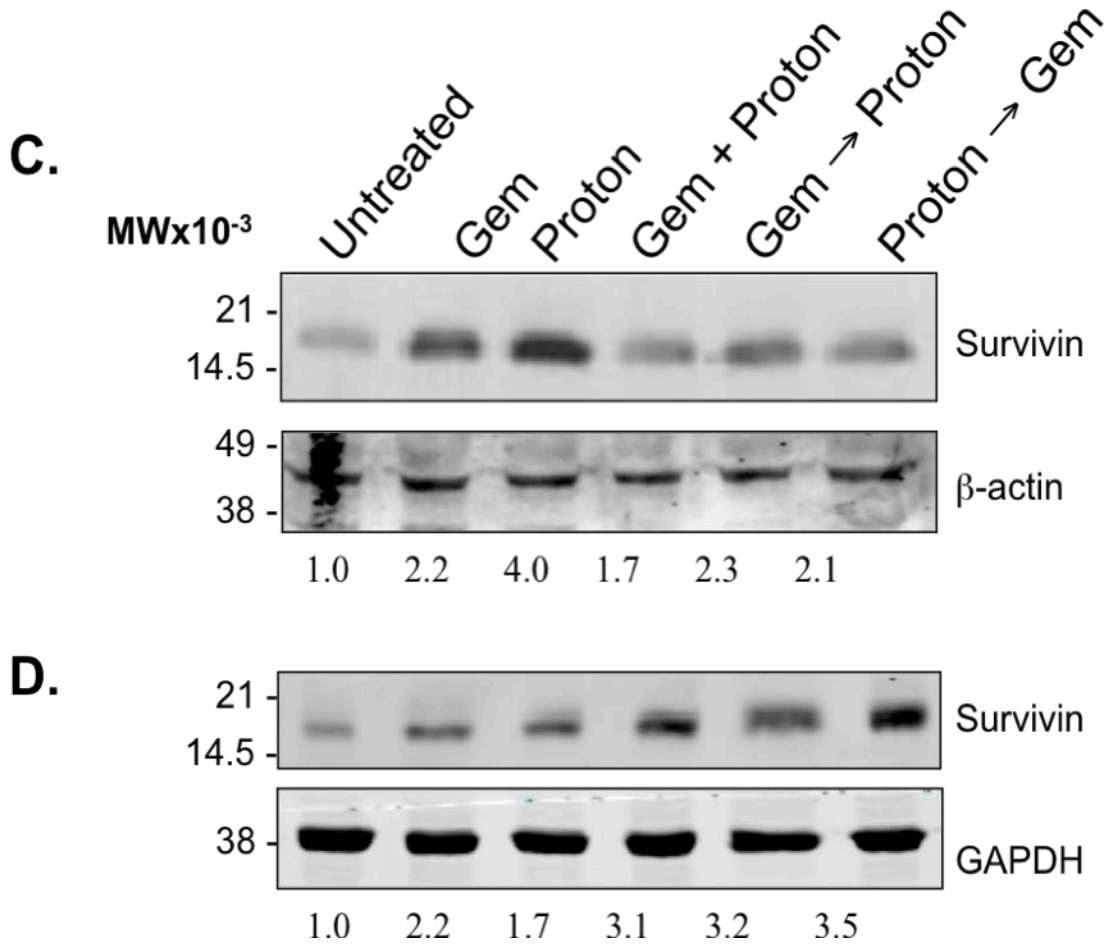

Figure 4 C & D. Detergent-solubilized extracts of **(C)** PANC-1 and **(D)** MIA PaCa-2 cells treated with 10 Gy of proton radiation and 10 μM gemcitabine were analyzed at 48h for reactivity with antibodies for survivin and β-actin or GAPDH (loading control), by Western blotting.

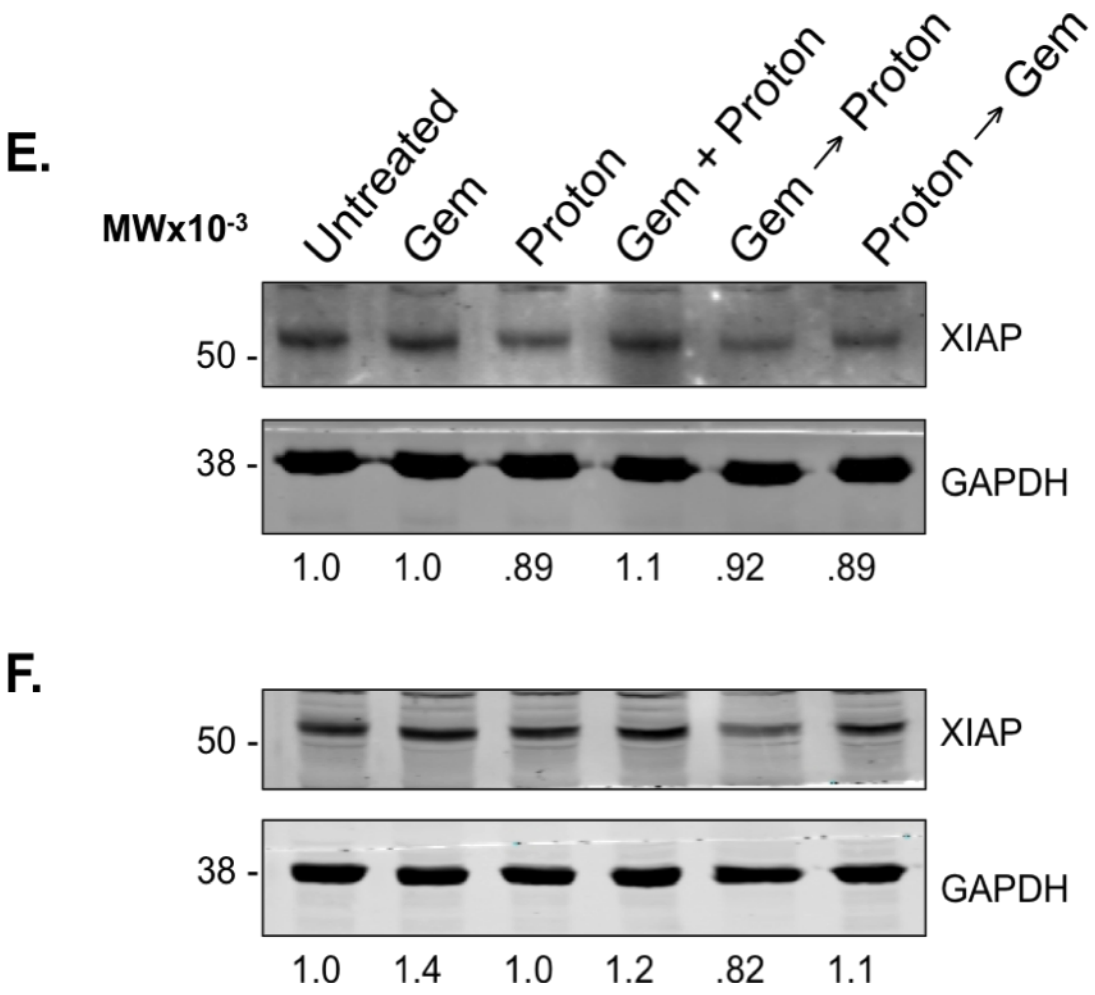

Figure 4 E & F: **(E)** PANC-1 and **(F)** MIA PaCa-2 membranes were stripped and reprobed with antibodies for XIAP after which densitometric fold changes for each were indicated below. Molecular-weight (M_r) markers in kilodaltons are shown on the left.

In contrast, treatment of MIA PaCa-2 cells only showed a 2 to 3 fold increase in those cells treated with protons. Gemcitabine treatment for 48 h resulted in a down regulation of survivin protein (Figure 4D). Both cell lines exhibited very little change in survivin protein expression from that of the control in the sequential combination treatments (Figure 4C, D). XIAP has been known to interact more directly with the apoptotic pathway machinery than survivin [135]. Both cell lines also exhibited very little change in XIAP protein expression compared to control, with the noticeable exception of gemcitabine → proton treatment (Figure 4E, F). This sequential treatment showed a marked decrease in XIAP protein expression, which may be responsible for the MIA PaCa-2 cells being more susceptible to the combination of gemcitabine and proton irradiation then the PANC-1 cells.

siRNA Knockdown of XIAP Further Potentiates Cell Death After Gemcitabine and Proton Combination Therapy

To further investigate the role survivin and XIAP may play in proton radiation resistance of the PANC-1 cells, siRNA knockdown experiments were completed. Three days after transfection with the siRNAs (described in Materials and Methods), cells were analyzed for the presence of Survivin and XIAP knockdown. PCR analysis indicated that survivin and XIAP knockdown was successful (Figure 5A), with approximately 75% knockdown of XIAP and 40% knockdown of Survivin.

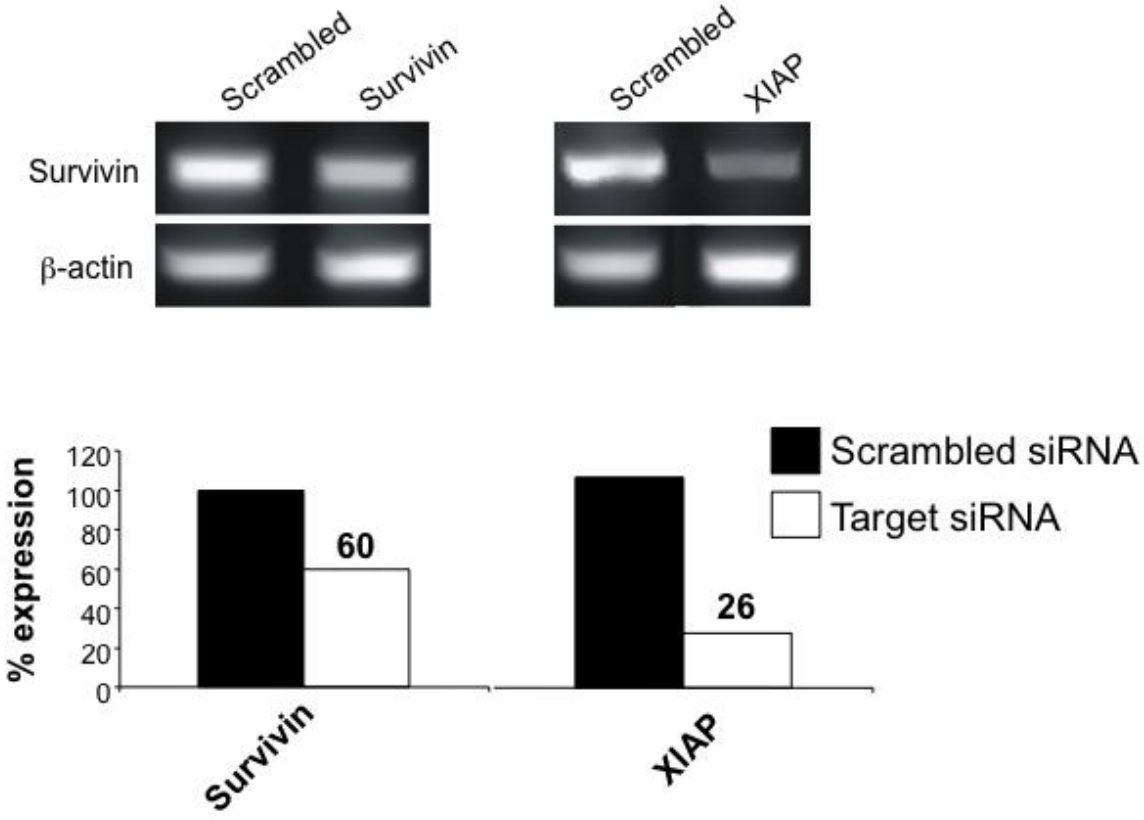

Figure 5. Knockdown of the inhibitor of apoptosis proteins survivin and XIAP, using siRNA, increases drug and radiation killing of PANC-1 pancreatic cancer cells. **(A)** Knockdown of survivin and XIAP expression. PANC-1 cells were transfected with either Scrambled siRNA or siRNA designed to knockdown survivin or XIAP.

Furthermore, after 72h IAP knockdown, cells were treated with either gemcitabine, proton radiation, or 24h gemcitabine followed by proton radiation (Figure 1). Forty-eight hours after treatment, cells were harvested for propidium iodide flow cytometric analysis. As hypothesized, the addition of the XIAP siRNA to the PANC-1 cells resulted in a marked increase in gemcitabine/proton-induced apoptosis compared to that recorded in the cells having survivin knockdown or those of the control (Figure 5B).

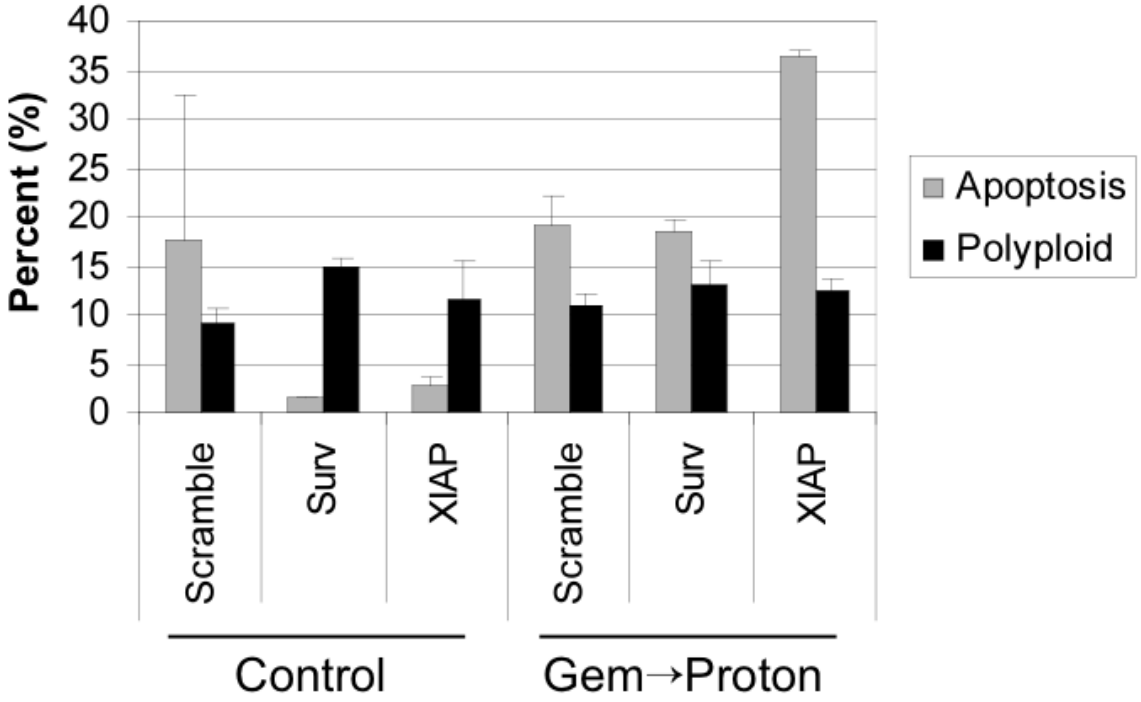

Figure 5B. (B) PANC-1 cells were first transfected with siRNA against either survivin or XIAP for 12 h after which they were treated using 10 Gy of proton radiation and 10 μM gemcitabine. Cells were harvested and analyzed for DNA content by propidium iodide staining and flow cytometry at 48 hr. Percentages of apoptotic cells with hypodiploid (sub-G1) DNA content as well as the polyploid are indicated per each condition tested. Data are the mean ± SD of two independent experiments.

Discussion

There has been little success in developing effective systemic therapies for the treatment of patients with locally advanced or metastatic pancreatic cancer. Chemotherapy was first combined with radiotherapy in the treatment of pancreatic cancer when clinicians at the Mayo Clinic in 1969 added 5-Fluorouracil (5-FU) to external beam radiotherapy. The result was an improved mean survival of 10.4 months for the combination therapy compared to 6.3 months for those patients receiving radiotherapy alone [136,137]. As a result, this combination has been considered standard therapy for locally advanced pancreatic cancer [137] and though multiagent regimens which include 5-FU have sought to improve upon this combination, randomized phase III trials have failed to confirm survival advantage over that with 5-FU alone [138]. More recently, the nucleoside analog gemcitabine provided encouraging results in both antitumor effects and its impact on parameters of clinical benefit for patients with pancreatic cancer such as, decreased pain severity, decreased requirement for opioid analgesics, increased appetite and weight gain [138]. In direct comparison on locally advanced pancreatic cancer and metastatic pancreatic cancer, gemcitabine treatment resulted in a 5.56 month overall survival compared to a 4.41 month overall survival using 5-FU. In combination with conventional radiotherapy gemcitabine extended overall survival to 11.3 months compared to 5-FU extending it by 10.4 months [137,138]. As a result, gemcitabine has become widely accepted for unresectable pancreatic cancer.

As pancreatic tumors are well advanced before detection, with survival reduced due to high rates of distant metastases, the continued use of conventional radiation-based therapies has been brought into question. As tumor loads increase, superfluous radiation delivered to surrounding normal tissue leads to increasing treatment morbidity. As a

result, better control of dose distribution and localization are necessary. Proton radiotherapy allows for both. Where local control is suboptimal with conventional photon radiotherapy, proton radiotherapy provides improved physical dose distribution, and improved localization to anatomic sites and histologies. The resulting improvements to both dose distribution and localization will ultimately lead to dose escalation for anatomical sites where local control with conventional radiation dose has been suboptimal such as in advanced pancreatic disease [125,139].

The aim of the current work was to define the involvement of survivin following chemotherapy and radiation therapy and to determine if proton irradiation followed classical radiation treatment observations. Our data shows that proton irradiation alone exhibited similar results as has been reported in photon radiation treatment. However, sequential treatment using gemcitabine before proton irradiation induced significant apoptotic cell death. While survivin seems to be minimally involved in the mechanism of radioresistance, our work provides evidence that XIAP down regulation may be involved in the sensitization of MIA PaCa-2 cells and the concomitant radioresistance of PANC-1 cells. It has been demonstrated that RNAi-mediated knockdown of XIAP as well as small molecule inhibitors of XIAP sensitize pancreatic cancer cells to apoptosis via activation of caspases 2, 3, 8 and 9, and loss of mitochondrial membrane polarization [140]. Furthermore, XIAP small molecule inhibitors have been shown to synergize the effects of radiation and gemcitabine alone [141].

An important finding of these studies was the treatment of PANC-1 and MIA PaCa-2 cells with proton irradiation caused a significant number of the cells to become polyploid. Polyploidy is a state in which cells possess more than two sets of homologous

chromosomes. It is commonly believed that polyploid cells arise after cellular stress, ageing, and in various diseases, perhaps because polyploidy confers a metabolic benefit [142-144]. Polyploid cells have been shown to be genetically unstable and can be the intermediates where aneuploid cells become cancerous [144]. In our hands, treatment of the pancreatic cancer cells lines with proton irradiation alone or before being combined with gemcitabine resulted in a significant enhanced polyploid fraction of cells (Figure 4). The cells treated with gemcitabine alone or with gemcitabine followed by proton irradiation prohibited this polyploidy. These findings suggest that proton irradiation-resistant pancreatic cells may gain enhanced genetic instability and ultimately a more aggressive tumor phenotype. However, administering gemcitabine as a pretreatment to proton irradiation may reduce this genetic instability and ultimately allow the proton irradiation to result in a more effective killing of the tumor. Furthermore, as polyploidy is a state of having more than two sets of chromosomes, survivin is a chromosomal passenger protein, and its deregulation in cancer promotes tetraploidy or aneuploidy, we strongly believe that by better understanding the role of gemcitabine and proton irradiation biology in regard to survivin expression modulation will provide useful data for the combining of therapies for the killing of radioresistant pancreatic cancer.

XIAP, a unique and best-characterized member of the inhibitor of apoptosis (IAP) family, has been identified as a central regulator of caspase-dependent apoptosis. Whether the activation of apoptosis is initiated by events that perturb the mitochondria (via caspase-9) or progress directly from cell surface receptors (via caspase-8), the ability of XIAP to inhibit the downstream executioner caspases-3 and -7 makes it a potent and broad inhibitor of cell death[145] and important target for therapy. XIAP reduction has

been reported in cells treated with protons and hypoxia in three kinds of cancers: lung, hepatoma and leukemia[146]. However, pancreatic cancers were not investigated. A broadened search to include photon and UV radiation revealed that much work has been accomplished investigating radiation-induced downregulation of XIAP and the mechanisms whereby this happens. A recent study describes UVB-induced sensitization coinciding with XIAP degradation which then allows for functional caspase 3-induced apoptosis[147]. Furthermore, the loss of XIAP was shown to be the result of UVB-enhanced Ikappa B alpha degradation, resulting in NF-kappaB-dependent transcriptional repression of XIAP[147]. Future studies will explore XIAP's involvement in the sequential chemo- and radiosensitization of pancreatic cancer as well as survivin's role in XIAP stabilization and the possibility of shifting the survival phenotype to apoptosis by interfering with this union.

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References

1. Kleeff, J.; Michalski, C.; Friess, H.; Buchler, M. W., Pancreatic cancer: from bench to 5-year survival. *Pancreas* **2006,** 33, (2), 111-8.

2. Bilimoria, K. Y.; Bentrem, D. J.; Ko, C. Y.; Stewart, A. K.; Winchester, D. P.; Talamonti, M. S., National failure to operate on early stage pancreatic cancer. *Ann Surg* **2007,** 246, (2), 173-80.

3. Muller, M. W.; Friess, H.; Koninger, J.; Martin, D.; Wente, M. N.; Hinz, U.; Ceyhan, G. O.; Blaha, P.; Kleeff, J.; Buchler, M. W., Factors influencing survival after bypass procedures in patients with advanced pancreatic adenocarcinomas. *Am J Surg* **2008,** 195, (2), 221-8.

4. Ueno, H.; Kiyosawa, K.; Kaniwa, N., Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy? *Br J Cancer* **2007,** 97, (2), 145-51.

5. Reni, M.; Cereda, S.; Galli, L., PEFG (cisplatin, epirubicin, 5-fluorouracil, gemcitabine) for patients with advanced pancreatic cancer: the ghost regimen. *Cancer Lett* **2007,** 256, (1), 25-8.

6. Oettle, H.; Neuhaus, P., Adjuvant therapy in pancreatic cancer: a critical appraisal. *Drugs* **2007,** 67, (16), 2293-310.

7. Olsen, D. R.; Bruland, O. S.; Frykholm, G.; Norderhaug, I. N., Proton therapy - a systematic review of clinical effectiveness. *Radiother Oncol* **2007,** 83, (2), 123-32.

8. Slater, J. D.; Yonemoto, L. T.; Mantik, D. W.; Bush, D. A.; Preston, W.; Grove, R. I.; Miller, D. W.; Slater, J. M., Proton radiation for treatment of cancer of the oropharynx: early experience at Loma Linda University Medical Center using a concomitant boost technique. *Int J Radiat Oncol Biol Phys* **2005,** 62, (2), 494-500.

9. Schulz-Ertner, D.; Tsujii, H., Particle radiation therapy using proton and heavier ion beams. *J Clin Oncol* **2007,** 25, (8), 953-64.

10. DeLaney, T. F., Clinical proton radiation therapy research at the Francis H. Burr Proton Therapy Center. *Technol Cancer Res Treat* **2007,** 6, (4 Suppl), 61-6.

11. Sarela, A. I.; Verbeke, C. S.; Ramsdale, J.; Davies, C. L.; Markham, A. F.; Guillou, P. J., Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma. *Br J Cancer* **2002,** 86, (6), 886-92.

12. Kami, K.; Doi, R.; Koizumi, M.; Toyoda, E.; Mori, T.; Ito, D.; Fujimoto, K.; Wada, M.; Miyatake, S.; Imamura, M., Survivin expression is a prognostic marker in pancreatic cancer patients. *Surgery* **2004,** 136, (2), 443-8.

13. Satoh, K.; Kaneko, K.; Hirota, M.; Masamune, A.; Satoh, A.; Shimosegawa, T., Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. *Cancer* **2001,** 92, (2), 271-8.

14. Li, F., Survivin study: what is the next wave? *J Cell Physiol* **2003,** 197, (1), 8-29.

15. Kim, K. W.; Mutter, R. W.; Willey, C. D.; Subhawong, T. K.; Shinohara, E. T.; Albert, J. M.; Ling, G.; Cao, C.; Gi, Y. J.; Lu, B., Inhibition of survivin and aurora B kinase sensitizes mesothelioma cells by enhancing mitotic arrests. *Int J Radiat Oncol Biol Phys* **2007,** 67, (5), 1519-25.

16. Asanuma, K.; Kobayashi, D.; Furuya, D.; Tsuji, N.; Yagihashi, A.; Watanabe, N., A role for survivin in radioresistance of pancreatic cancer cells. *Jpn J Cancer Res* **2002,** 93, (9), 1057-62.

17. Mesri, M.; Wall, N. R.; Li, J.; Kim, R. W.; Altieri, D. C., Cancer gene therapy using a survivin mutant adenovirus. *J Clin Invest* **2001,** 108, (7), 981-90.

18. Pennati, M.; Binda, M.; Colella, G.; Folini, M.; Citti, L.; Villa, R.; Daidone, M. G.; Zaffaroni, N., Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression. *J Invest Dermatol* **2003,** 120, (4), 648-54.

19. Kami, K.; Doi, R.; Koizumi, M.; Toyoda, E.; Mori, T.; Ito, D.; Kawaguchi, Y.; Fujimoto, K.; Wada, M.; Miyatake, S.; Imamura, M., Downregulation of survivin by siRNA diminishes radioresistance of pancreatic cancer cells. *Surgery* **2005,** 138, (2), 299-305.

20. Lee, J. U.; Hosotani, R.; Wada, M.; Doi, R.; Kosiba, T.; Fujimoto, K.; Miyamoto, Y.; Tsuji, S.; Nakajima, S.; Nishimura, Y.; Imamura, M., Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. *Eur J Cancer* **1999,** 35, (9), 1374-80.

21. Li, F.; Ackermann, E. J.; Bennett, C. F.; Rothermel, A. L.; Plescia, J.; Tognin, S.; Villa, A.; Marchisio, P. C.; Altieri, D. C., Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol* **1999,** 1, (8), 461-6.

22. O'Connor, D. S.; Wall, N. R.; Porter, A. C.; Altieri, D. C., A p34(cdc2) survival checkpoint in cancer. *Cancer Cell* **2002,** 2, (1), 43-54.

23. Dohi, T.; Xia, F.; Altieri, D. C., Compartmentalized phosphorylation of IAP by protein kinase A regulates cytoprotection. *Mol Cell* **2007,** 27, (1), 17-28.

24. Moertel, C. G.; Childs, D. S., Jr.; Reitemeier, R. J.; Colby, M. Y., Jr.; Holbrook, M. A., Combined 5-fluorouracil and supervoltage radiation therapy of locally unresectable gastrointestinal cancer. *Lancet* **1969,** 2, (7626), 865-7.

25. Park, J. K.; Ryu, J. K.; Lee, J. K.; Yoon, W. J.; Lee, S. H.; Kim, Y. T.; Yoon, Y. B., Gemcitabine chemotherapy versus 5-fluorouracil-based concurrent chemoradiotherapy in locally advanced unresectable pancreatic cancer. *Pancreas* **2006,** 33, (4), 397-402.

26. Burris, H. A., 3rd; Moore, M. J.; Andersen, J.; Green, M. R.; Rothenberg, M. L.; Modiano, M. R.; Cripps, M. C.; Portenoy, R. K.; Storniolo, A. M.; Tarassoff, P.; Nelson, R.; Dorr, F. A.; Stephens, C. D.; Von Hoff, D. D., Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* **1997,** 15, (6), 2403-13.

27. Trofimov, A.; Nguyen, P. L.; Coen, J. J.; Doppke, K. P.; Schneider, R. J.; Adams, J. A.; Bortfeld, T. R.; Zietman, A. L.; Delaney, T. F.; Shipley, W. U., Radiotherapy treatment of early-stage prostate cancer with IMRT and protons: a treatment planning comparison. *Int J Radiat Oncol Biol Phys* **2007,** 69, (2), 444-53.

28. Giagkousiklidis, S.; Vellanki, S. H.; Debatin, K. M.; Fulda, S., Sensitization of pancreatic carcinoma cells for gamma-irradiation-induced apoptosis by XIAP inhibition. *Oncogene* **2007,** 26, (49), 7006-16.

29. Karikari, C. A.; Roy, I.; Tryggestad, E.; Feldmann, G.; Pinilla, C.; Welsh, K.; Reed, J. C.; Armour, E. P.; Wong, J.; Herman, J.; Rakheja, D.; Maitra, A., Targeting the apoptotic machinery in pancreatic cancers using small-molecule antagonists of the X-linked inhibitor of apoptosis protein. *Mol Cancer Ther* **2007,** 6, (3), 957-66.

30. Comai, L., The advantages and disadvantages of being polyploid. *Nat Rev Genet* **2005,** 6, (11), 836-46.

31. Storchova, Z.; Pellman, D., From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* **2004,** 5, (1), 45-54.

32. Ganem, N. J.; Storchova, Z.; Pellman, D., Tetraploidy, aneuploidy and cancer. *Curr Opin Genet Dev* **2007,** 17, (2), 157-62.

33. Mufti, A. R.; Burstein, E.; Duckett, C. S., XIAP: cell death regulation meets copper homeostasis. *Arch Biochem Biophys* **2007,** 463, (2), 168-74.

34. Lee, K. B.; Kim, K. R.; Huh, T. L.; Lee, Y. M., Proton induces apoptosis of hypoxic tumor cells by the p53-dependent and p38/JNK MAPK signaling pathways. *Int J Oncol* **2008,** 33, (6), 1247-56.

35. Thayaparasingham, B.; Kunz, A.; Peters, N.; Kulms, D., Sensitization of melanoma cells to TRAIL by UVB-induced and NF-kappaB-mediated downregulation of xIAP. *Oncogene* **2009,** 28, (3), 345-62.

CHAPTER FOUR

DISCUSSION

This work demonstrates a role for YY1 in the transcriptional regulation of survivin, an important target in cancer therapeutics. Contrary to our initial hypothesis, YY1 was shown to downregulate survivin promoter activity. The second part of this work shows that, at least in principle, decreased expression of IAPs such as survivin and XIAP through a combination of chemotherapy with proton radiotherapy may increase the sensitivity of tumors to cell death. While the latter aspect of the dissertation has a clear implication in cancer therapy, the former aspect may draw criticism from the cancer biology community owing to its lack of application to the specific approaches to cancer treatment. In the past this criticism may have been more relevant, but current approaches to cancer therapy are beginning to include transcription factors as very valid targets of cancer therapy. As previously mentioned, Hanahan and Weinberg have produced seminal work summarizing the hallmarks of cancer [148]. These hallmarks include (1) evasion of programmed cell death, (2) insensitivity to growth-inhibitory signals, (3) limitless replicative potential, (4) sustained angiogenesis, (5) self-sufficiency in growth signals, and (6) tissue invasion and metastatic spread. They more recently proposed two emerging hallmarks that include 1) evasion of immune destruction and 2) deregulation of cellular energetics [149]. A review by Mees et al. has eloquently summarized how a variety of transcription factors play direct roles in each of these hallmarks of cancer in hopes of furthering a shift in thinking that embraces targeting of transcription factors in cancer

therapy [150]. Among the many transcription factors discussed, several are worth noting in greater detail here given their relevance to survivin and YY1. Table 3 summarizes several transcription factors with specific relevance to survivin and YY1.

Table 3: Transcription Factors Involvement in Hallmarks of Cancer and Their Relationship to Survivin and YY1 (adapted from Mees et al. [150]).

Transcription Factor/Target	Hallmark of Cancer	Rationale
NF-κB	Sufficiency in growth signals	Constiutively active in many cancers. Positively regulates survivin transcription.
Androgen receptor	Sufficiency in growth signals	YY1 directly interacts with AR and enhances AR interaction with PSA promoter.
Myc	Insensitivity to growth-inhibitory signals	YY1 activates c-myc promoter
p53	Evasion of programmed cell death	Survivin downregulated by p53. YY1 downregulates p53.
HIF-1α	Sustained angiogenesis	Positively regulates survivin transcription
Sp-1	Sustained angiogenesis/evasion of programmed cell death	Transcriptional activator of survivin. Interruption of Sp-1 binding to survivin promoter induces cell death

Therapeutic Potential of YY1 in Cancer Therapy

The role of survivin in therapeutic approaches to cancer remains promising, as there are ongoing efforts to target it in new and innovative ways. Although the role of YY1 in cancer has been known for a while now, efforts to exploit it for therapy are in their relative infancy. Just as the role of YY1 in cancer biology is controversial, so is its value in therapeutic approaches to cancer. In ovarian cancer patients, one group found a positive correlation between YY1 expression and response to taxane therapy. In this study, YY1 knockdown lead to a significant reduction in cell proliferation and anchorage-independent growth as well as increased effectiveness of the drug paclitaxel [78]. This is postulated to be because of positive regulation of genes involved in microtubule stabilizing activity. TRAIL is a promising ligand for inducing cell death in clinical applications because it has been shown to induce anti-tumor activity while sparing nonmalignant tissue [151]. TRAIL induces cell death by binding to death receptors DR4 or DR5 with subsequent activation of caspases. Baritaki et al. [152] showed that siRNA-mediated knockdown of YY1 results in increased DR5 expression and sensitization to TRAIL-mediated apoptosis. YY1 is capable of directly binding to the DR5 promoter to downregulate its expression [153]. They also show that treatment of prostate cancer cells (PC3) with the nitric oxide donor DETANONOate sensitizes cells to TRAIL-induced cell death by downregulating NFKB and downstream of that YY1. The same group has shown that inhibition of the anti-apoptotic factor BCL_{XL} is also involved, but it is unknown if this is via regulation of BCL_{XL} by YY1. This represents another line of evidence that interruption of YY1 activity has potential for sensitization of tumors to chemotherapy and other treatment modalities. Given the controversy as to YY1's role in

cancer, this will result in significant challenges in understanding how to approach individual types of cancer therapy as it relates to inhibition of YY1. This will require that targeting of YY1 be highly cancer specific to avoid dysregulation of YY1 in normal tissue.

YY1 and Survivin: Beyond Transcriptional Regulation

Tumor metastasis is the most common cause of death in cancer patients. It is a remarkably complex process with several several steps described by Hanahan and Weinberg that are required for a malignant cell to fully metastasize [148]. The first step is invasion, which involves the loss of cell adhesion molecules. Without loss of surface adhesion molecules it is not possible for a cell to begin migration into neighboring tissue. The second step involves intravasation of the invading malignant cells into the blood or lymphatic system. The third step, which only a small percent of intravasated cells are though to be able to accomplish, is extravasation through capillaries at a site distant to the primary tumor. Once extravasated, cells must then regain adhesion molecules that allow the cell to establish the ability to survive in the new environment. In carcinomas, the metastatic process is thought to consist of a number of distinct steps. The complex mechanisms required to accomplish each of these steps are the subject of intense research.

Epithelial-to-Mesenchymal Transition (EMT) is a proposed mechanism by which malignant cells initiate the need for a loss of adhesion molecules so that invasion can take place. EMT is characterized by a loss of E-cadherin and β-catenin, and a gain in N-cadherin and vimentin expression (reviewed by Huber et al. [154]). The transcription factors Twist, Snail, and Slug have been identified as the major regulators of these

adhesion molecules [155-157]. Research from our laboratory indicates that survivin is involved in the invasion step of metastasis [158].

Survivin is overexpressed in primary tumors in addition to distant metastatic cells, however no direct involvement in the mechanism of metastasis has been identified. Our lab has recently found a novel means by which survivin promotes cell invasion. Extracellular survivin is able to promote invasion of HeLa cells through a collagen matrix, and antibody depletion of survivin abrogates this increased cell invasion [158]. Studies are ongoing to identify mechanisms behind this observation. Very recently Yie et al. [159] found that patients with survivin-expressing circulating non-small cell lung cancer cells had a higher instance of cancer recurrence and increased follow-up lymph node involvement. Other studies have shown that survivin is able to delineate node positive from node negative rectal cancer [160]. In small cell adencarcinoma of the lung, patients with histological evidence of high survivin expression had more evidence of veinous invasion of malignant cells, and overall patients with high survivin expression had decreased survival [161]. In squamous cell carcinomas, survivin expression correlates with high grade, poorly differentiated tumors and with increased lymph node metastasis [162]. A common theme in these studies is the presence of almost entirely correlative data with little or no mechanistic information.

Among the many target genes of YY1 being discovered, some involved in metastasis are now being identified. A report in 2005 hypothesized that cooperation of YY1 and AP-1 may increase the repression of the galactocerebrosidase (GALC) gene. GALC is an enzyme that is overexpressed on the surface of cancer cells. Suppression of this enzyme leads to an accumulation of galactocerebroside, which results in a decrease

in cellular adhesion and inhibition of apoptosis. This in turn leads to increased cell proliferation and migration [163]. This observation, although it was largely conjecture, was the first evidence that YY1 may be involved in cancer invasion and metastasis. However, in the search to identify new genes involved in metastasis suppression, Wang et al. (2005) discovered that HLJ1, a metastasis suppressor, is positively regulated by YY1 [164]. High levels of YY1 expression correlated with HLJ1 expression , and promoter reporter assays indicated that YY1 was acting directly on transcription of HLJ1. Subsequent studies found that a synergistic relationship between YY1 and AP1 lead to a 5 times higher activation of HLJ1 and much more potent *in vitro* cancer cell invasion [165]. Using the osteosarcoma cell line SaOS-2, de Negris et al.[64] found that deletion of YY1 leads to a decrease in cell invasion *in vitro* and decrease metastasis *in vivo*. Deletion of YY1 also correlated with a decrease in vascular endothelial growth factor (VEGF) and angiogenesis. They also identified a host of genes involved in cell motility, cell cycle, cell adhesion, angiogenesis, and signal transduction that exhibited significant changes when YY1 was deleted [64]. One report suggested that YY1 is a regulator of Snail, one of the key transcription factors responsible for regulation of EMT, a key feature of metastasis [166]. Together these data detail the complicated nature of YY1's involvement in cancer metastasis, as it appears that in some types of cancer it may inhibit metastasis, while in others such as osteosarcoma it may promote metastasis and aggressiveness of the disease.

Future Directions

The work presented here illustrates a role for YY1 in survivin transcriptional repression in the osteosarcoma cell line U2OS. However, the role of YY1 in

transcription of survivin in other cancer types has yet to be thoroughly investigated. Preliminary evidence from our laboratory indicates a similar repressive role for YY1's observed repression of survivin in the pancreatic cancer cell line Panc-1. In Chapter 3, data was presented that indicated an important role for survivin expression levels in Panc-1 radioresistance, but it suggested an even larger role for the inhibitor of apoptosis XIAP in the radioresistance of PANC-1 cells. Therefore, future studies should broaden the investigation of cancer-specific YY1 regulation of survivin transcription and be expanded to investigation of transcriptional regulation of IAPs such as XIAP.

The work presented in Chapter 2 shows multiple avenues of evidence for the involvement of YY1 involvement in basal survivin transcription, but future efforts should attempt to identify the role of YY1 in cellular response to stresses in the form of chemotherapeutics, radiotherapy, or natural agent exposure. HIF-1α's role in survivin transcriptional upregulation is now well established 30,31. While YY1 is not clearly established as a stress-response transcriptional factor per se, several studies have indicated that is involved in unfolded protein response and resulting ER stress 167 and may even inhibit the function of p53 in response to genotoxic stress 168.

In keeping with the need to better understand YY1's role in survivin-mediated cellular stress response, it is also critical for future studies to measure functional outcomes as a consequence of survivin transcriptional modulation. Preliminary evidence in our lab indicates that YY1 overexpression in U2OS tet-off cells may be involved in enhanced cellular proliferation as measured by the Ki-67 assay (unpublished data). However, it is unknown the extent to which YY1 overexpression is specifically involved in this enhanced proliferation or if it is indeed mediated by survivin or by one of the other

numerous transcriptional targets of YY1. The reporter data presented in Chapter 2 indicates very robust repression of survivin promoter activity when YY1 is overexpressed, but much more moderate reduction in protein expression. A recent study was able to show a role for YY1 in mammary cell proliferation, migration, clonogenicity, invasion, and tumor formation, and they identified YY1-mediated p27 degradation as a likely mechanism behind this 169. In a similar fashion, future work should elucidate whether YY1 is able to modulate cellular invasion, migration, proliferation, and other outcomes through its regulation of survivin. These studies will be critical to further efforts to establish new therapeutic approaches based on survivin targeting.

Our lab has recently described a novel pool of survivin existing in the extracellular space [158]. Current studies are exploring ways in which this pool of survivin may contribute to disease in the normal neighboring cells in the tumor microenvironment. Antibody depletion of this extracellular pool of survivin may prove to be a valid therapeutic approach for solid tumors. However, at this point the mechanism for export of survivin in unknown and under investigation. If YY1 is indeed a modulator of survivin transcription, it stands to reason that YY1 overexpression or knockdown may alter the amount of survivin that is exported to the extracellular space.

Summary and Conclusion

Cancer is a disease that is increasingly being understood to be a constellation of hundreds if not thousands of different diseases. This is likely why, despite a multitude of significant advances in our understanding of cancer, current therapies leave much to be desired in terms of patient health and well being. The future of cancer therapy will hopefully include personalized approaches to individual disease, but this will require a

more complete understanding of the underlying factors involved in cancer development and advancement. The work described in this dissertation will serve as a small, but perhaps important, addition to the body of knowledge regarding survivin transcription and the role of survivin and other IAPs in resistance to death induction by presently used chemotherapeutics and radiotherapy techniques.

REFERENCES

1. Tarver T. Cancer Facts & Figures 2012. American Cancer Society (ACS). Journal of Consumer Health on the Internet 2012;16:366-7.

2. Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 2003;22:8581-9.

3. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580-4.

4. Altieri DC. The case for survivin as a regulator of microtubule dynamics and cell-death decisions. Curr Opin Cell Biol 2006;18:609-15.

5. Fengzhi Li XL. Survivin study: An update of ldquoWhat is the next wave?rdquo. Journal of Cellular Physiology 2006;208:476-86.

6. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917-21.

7. Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. Genes Dev 1999;13:239-52.

8. Dohi T, Beltrami E, Wall NR, Plescia J, Altieri DC. Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. J Clin Invest 2004;114:1117-27.

9. Fortugno P, Wall NR, Giodini A, et al. Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function. J Cell Sci 2002;115:575-85.

10. Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC. Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. Am J Pathol 1998;152:43-9.

11. Fukuda S, Pelus LM. Survivin, a cancer target with an emerging role in normal adult tissues. Mol Cancer Ther 2006;5:1087-98.

12. Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, thor Straten P. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. Cancer Res 2001;61:5964-8.

13. Grossman D, Kim PJ, Schechner JS, Altieri DC. Inhibition of melanoma tumor growth in vivo by survivin targeting. Proc Natl Acad Sci U S A 2001;98:635-40.

14. Mesri M, Wall NR, Li J, Kim RW, Altieri DC. Cancer gene therapy using a survivin mutant adenovirus. J Clin Invest 2001;108:981-90.

15. Wall NR, O'Connor DS, Plescia J, Pommier Y, Altieri DC. Suppression of survivin phosphorylation on Thr34 by flavopiridol enhances tumor cell apoptosis. Cancer Res 2003;63:230-5.

16. Plescia J, Salz W, Xia F, et al. Rational design of shepherdin, a novel anticancer agent. Cancer Cell 2005;7:457-68.

17. Olie RA, Simoes-Wust AP, Baumann B, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000;60:2805-9.

18. Kanwar JR, Shen WP, Kanwar RK, Berg RW, Krissansen GW. Effects of survivin antagonists on growth of established tumors and B7-1 immunogene therapy. J Natl Cancer Inst 2001;93:1541-52.

19. Andersen MH, Svane IM, Becker JC, Straten PT. The universal character of the tumor-associated antigen survivin. Clin Cancer Res 2007;13:5991-4.

20. Mera S, Magnusson M, Tarkowski A, Bokarewa M. Extracellular survivin up-regulates adhesion molecules on the surface of leukocytes changing their reactivity pattern. J Leukoc Biol 2008;83:149-55.

21. Bokarewa M, Lindblad S, Bokarew D, Tarkowski A. Balance between survivin, a key member of the apoptosis inhibitor family, and its specific antibodies determines erosivity in rheumatoid arthritis. Arthritis Res Ther 2005;7:R349-58.

22. Khan S, Jutzy JM, Aspe JR, McGregor DW, Neidigh JW, Wall NR. Survivin is released from cancer cells via exosomes. Apoptosis : an international journal on programmed cell death 2011;16:1-12.

23. Vaux DL, Korsmeyer SJ. Cell death in development. Cell 1999;96:245-54.

24. Velculescu VE, Madden SL, Zhang L, et al. Analysis of human transcriptomes. Nat Genet 1999;23:387-8.

25. Bao R, Connolly DC, Murphy M, et al. Activation of Cancer-Specific Gene Expression by the Survivin Promoter. J Natl Cancer Inst 2002;94:522-8.

26. Zhu ZB, Makhija SK, Lu B, et al. Transcriptional targeting of tumors with a novel tumor-specific survivin promoter. Cancer Gene Ther 2004;11:256-62.

27. Lu B, Makhija SK, Nettelbeck DM, et al. Evaluation of tumor-specific promoter activities in melanoma. Gene Ther 2005;12:330-8.

28. Wei H, Wang C, Chen L. Proliferating cell nuclear antigen, survivin, and CD34 expressions in pancreatic cancer and their correlation with hypoxia-inducible factor 1alpha. Pancreas 2006;32:159-63.

29. Chang Q, Qin R, Huang T, Gao J, Feng Y. Effect of antisense hypoxia-inducible factor 1alpha on progression, metastasis, and chemosensitivity of pancreatic cancer. Pancreas 2006;32:297-305.

30. Peng XH, Karna P, Cao Z, Jiang BH, Zhou M, Yang L. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. J Biol Chem 2006;281:25903-14.

31. Bai H, Ge S, Lu J, Qian G, Xu R. Hypoxia inducible factor-1alpha-mediated activation of survivin in cervical cancer cells. The journal of obstetrics and gynaecology research 2013;39:555-63.

32. Yun YJ, Li SH, Cho YS, Park JW, Chun YS. Survivin mediates prostate cell protection by HIF-1alpha against zinc toxicity. Prostate 2010;70:1179-88.

33. Chen YQ, Zhao CL, Li W. Effect of hypoxia-inducible factor-1alpha on transcription of survivin in non-small cell lung cancer. J Exp Clin Cancer Res 2009;28:29.

34. Li DW, Zhou L, Jin B, Xie J, Dong P. Expression and significance of hypoxia-inducible factor-1alpha and survivin in laryngeal carcinoma tissue and cells. Otolaryngol Head Neck Surg 2013;148:75-81.

35. Fan LF, Dong WG, Jiang CQ, Qian Q, Yu QF. Role of Hypoxia-inducible factor-1 alpha and Survivin in colorectal carcinoma progression. International journal of colorectal disease 2008;23:1057-64.

36. Li F, Altieri DC. Transcriptional analysis of human survivin gene expression. Biochem J 1999;344 Pt 2:305-11.

37. Zhu N, Gu L, Findley HW, et al. KLF5 Interacts with p53 in regulating survivin expression in acute lymphoblastic leukemia. J Biol Chem 2006;281:14711-8.

38. Filippo F. Angileri MHAACDLTSCRCCTAGGVFT. Nuclear factor-kappaB activation and differential expression of survivin and Bcl-2 in human grade 2-4 astrocytomas. Cancer 2008;112:2258-66.

39. Zucchini C, Rocchi A, Manara MC, et al. Apoptotic genes as potential markers of metastatic phenotype in human osteosarcoma cell lines. Int J Oncol 2008;32:17-31.

40. Hirochika Kawakami MTTMTOYTMFMHTTNM. Transcriptional activation of survivin through the NF-kappaB pathway by human T-cell leukemia virus type I tax. International Journal of Cancer 2005;115:967-74.

41. Gritsko T, Williams A, Turkson J, et al. Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. Clin Cancer Res 2006;12:11-9.

42. Raj D, Liu T, Samadashwily G, Li F, Grossman D. Survivin repression by p53, Rb and E2F2 in normal human melanocytes. Carcinogenesis 2008;29:194-201.

43. Ackerman SL, Minden AG, Williams GT, Bobonis C, Yeung CY. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. Proc Natl Acad Sci U S A 1991;88:7523-7.

44. Skerka C, Decker EL, Zipfel PF. A regulatory element in the human interleukin 2 gene promoter is a binding site for the zinc finger proteins Sp1 and EGR-1. J Biol Chem 1995;270:22500-6.

45. Dong X-P, Pfister H. Overlapping YY1- and aberrant SP1-binding sites proximal to the early promoter of human papillomavirus type 16. J Gen Virol 1999;80:2097-101.

46. Cheson BD, Bartlett NL, Vose JM, et al. A phase II study of the survivin suppressant YM155 in patients with refractory diffuse large B-cell lymphoma. Cancer 2012;118:3128-34.

47. Tolcher AW, Quinn DI, Ferrari A, et al. A phase II study of YM155, a novel small-molecule suppressor of survivin, in castration-resistant taxane-pretreated prostate cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 2012;23:968-73.

48. Lewis KD, Samlowski W, Ward J, et al. A multi-center phase II evaluation of the small molecule survivin suppressor YM155 in patients with unresectable stage III or IV melanoma. Investigational new drugs 2011;29:161-6.

49. Giaccone G, Zatloukal P, Roubec J, et al. Multicenter phase II trial of YM155, a small-molecule suppressor of survivin, in patients with advanced, refractory, non-small-cell lung cancer. J Clin Oncol 2009;27:4481-6.

50. Nakahara T, Kita A, Yamanaka K, et al. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. Cancer Res 2007;67:8014-21.

51. Nakamura N, Yamauchi T, Hiramoto M, et al. Interleukin enhancer-binding factor 3/NF110 is a target of YM155, a suppressant of survivin. Mol Cell Proteomics 2012;11:M111 013243.

52. Gandhy SU, Kim K, Larsen L, Rosengren RJ, Safe S. Curcumin and synthetic analogs induce reactive oxygen species and decreases specificity protein (Sp) transcription factors by targeting microRNAs. BMC Cancer 2012;12:564.

53. Qiao Q, Jiang Y, Li G. Inhibition of the PI3K/AKT-NF-kappaB Pathway With Curcumin Enhanced Radiation-Induced Apoptosis in Human Burkitt's Lymphoma. Journal of pharmacological sciences 2013;121:247-56.

54. Qiao Q, Jiang Y, Li G. Curcumin enhances the response of non-Hodgkin's lymphoma cells to ionizing radiation through further induction of cell cycle arrest at the G2/M phase and inhibition of mTOR phosphorylation. Oncol Rep 2013;29:380-6.

55. Del Follo-Martinez A, Banerjee N, Li X, Safe S, Mertens-Talcott S. Resveratrol and Quercetin in Combination Have Anticancer Activity in Colon Cancer Cells and Repress Oncogenic microRNA-27a. Nutrition and cancer 2013;65:494-504.

56. Ficzycz A, Ovsenek N. The Yin Yang 1 transcription factor associates with ribonucleoprotein (mRNP) complexes in the cytoplasm of Xenopus oocytes. J Biol Chem 2002;277:8382-7.

57. Galvin KM, Shi Y. Multiple mechanisms of transcriptional repression by YY1. Mol Cell Biol 1997;17:3723-32.

58. Shi Y, Seto E, Chang LS, Shenk T. Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. Cell 1991;67:377-88.

59. Yao YL, Yang WM, Seto E. Regulation of transcription factor YY1 by acetylation and deacetylation. Mol Cell Biol 2001;21:5979-91.

60. Chang LS, Shi Y, Shenk T. Adeno-associated virus P5 promoter contains an adenovirus E1A-inducible element and a binding site for the major late transcription factor. J Virol 1989;63:3479-88.

61. Shi Y, Lee JS, Galvin KM. Everything you have ever wanted to know about Yin Yang 1. Biochim Biophys Acta 1997;1332:F49-66.

62. Donohoe ME, Zhang X, McGinnis L, Biggers J, Li E, Shi Y. Targeted disruption of mouse Yin Yang 1 transcription factor results in peri-implantation lethality. Mol Cell Biol 1999;19:7237-44.

63. Affar EB, Gay F, Shi Y, et al. Essential Dosage-Dependent Functions of the Transcription Factor Yin Yang 1 in Late Embryonic Development and Cell Cycle Progression. Mol Cell Biol 2006;26:3565-81.

64. de Nigris F, Rossiello R, Schiano C, et al. Deletion of Yin Yang 1 protein in osteosarcoma cells on cell invasion and CXCR4/angiogenesis and metastasis. Cancer Res 2008;68:1797-808.

65. Chan YJ, Chiou CJ, Huang Q, Hayward GS. Synergistic interactions between overlapping binding sites for the serum response factor and ELK-1 proteins mediate both basal enhancement and phorbol ester responsiveness of primate cytomegalovirus major immediate-early promoters in monocyte and T-lymphocyte cell types. J Virol 1996;70:8590-605.

66. Garban HJ, Bonavida B. Nitric oxide inhibits the transcription repressor Yin-Yang 1 binding activity at the silencer region of the Fas promoter: a pivotal role for nitric oxide in the up-regulation of Fas gene expression in human tumor cells. J Immunol 2001;167:75-81.

67. Vega MI, Jazirehi AR, Huerta-Yepez S, Bonavida B. Rituximab-induced inhibition of YY1 and Bcl-xL expression in Ramos non-Hodgkin's lymphoma cell line via inhibition of NF-kappa B activity: role of YY1 and Bcl-xL in Fas resistance and chemoresistance, respectively. J Immunol 2005;175:2174-83.

68. Castellano G, Torrisi E, Ligresti G, et al. The involvement of the transcription factor Yin Yang 1 in cancer development and progression. Cell Cycle 2009;8:1367-72.

69. Deng Z, Cao P, Wan MM, Sui G. Yin Yang 1: a multifaceted protein beyond a transcription factor. Transcription 2010;1:81-4.

70. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. Oncogene 2005;25:1125-42.

71. Seligson D, Horvath S, Huerta-Yepez S, et al. Expression of transcription factor Yin Yang 1 in prostate cancer. Int J Oncol 2005;27:131-41.

72. Deng Z, Wan M, Cao P, Rao A, Cramer SD, Sui G. Yin Yang 1 regulates the transcriptional activity of androgen receptor. Oncogene 2009;28:3746-57.

73. Baritaki S, Sifakis S, Huerta-Yepez S, et al. Overexpression of VEGF and TGF-beta1 mRNA in Pap smears correlates with progression of cervical intraepithelial neoplasia to cancer: implication of YY1 in cervical tumorigenesis and HPV infection. Int J Oncol 2007;31:69-79.

74. Yakovleva T, Kolesnikova L, Vukojevic V, et al. YY1 binding to a subset of p53 DNA-target sites regulates p53-dependent transcription. Biochem Biophys Res Commun 2004;318:615-24.

75. Sui G, Affar el B, Shi Y, et al. Yin Yang 1 is a negative regulator of p53. Cell 2004;117:859-72.

76. Lane DP. p53 and human cancers. British medical bulletin 1994;50:582-99.

77. Berchuck A, Iversen ES, Lancaster JM, et al. Patterns of gene expression that characterize long-term survival in advanced stage serous ovarian cancers. Clin Cancer Res 2005;11:3686-96.

78. Matsumura N, Huang Z, Baba T, et al. Yin yang 1 modulates taxane response in epithelial ovarian cancer. Mol Cancer Res 2009;7:210-20.

79. de Nigris F, Botti C, de Chiara A, et al. Expression of transcription factor Yin Yang 1 in human osteosarcomas. European Journal of Cancer 2006;42:2420-4.

80. Grubach L, Juhl-Christensen C, Rethmeier A, et al. Gene expression profiling of Polycomb, Hox and Meis genes in patients with acute myeloid leukaemia. Eur J Haematol 2008;81:112-22.

81. Sakhinia E, Glennie C, Hoyland JA, et al. Clinical quantitation of diagnostic and predictive gene expression levels in follicular and diffuse large B-cell lymphoma by RT-PCR gene expression profiling. Blood 2007;109:3922-8.

82. Naidoo K, Clay V, Hoyland JA, et al. YY1 expression predicts favourable outcome in follicular lymphoma. J Clin Pathol 2011;64:125-9.

83. Goldstein D, Van Hazel G, Walpole E, et al. Gemcitabine with a specific conformal 3D 5FU radiochemotherapy technique is safe and effective in the definitive management of locally advanced pancreatic cancer. Br J Cancer 2007;97:464-71.

84. Satoh K, Kaneko K, Hirota M, Masamune A, Satoh A, Shimosegawa T. Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. Cancer 2001;92:271-8.

85. Terashima K, Demizu Y, Hashimoto N, et al. A phase I/II study of gemcitabine-concurrent proton radiotherapy for locally advanced pancreatic cancer without distant metastasis. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 2012;103:25-31.

86. Bush DA, Slater JD, Garberoglio C, Yuh G, Hocko JM, Slater JM. A technique of partial breast irradiation utilizing proton beam radiotherapy: comparison with conformal x-ray therapy. Cancer journal 2007;13:114-8.

87. Ronson BB, Schulte RW, Han KP, Loredo LN, Slater JM, Slater JD. Fractionated proton beam irradiation of pituitary adenomas. Int J Radiat Oncol Biol Phys 2006;64:425-34.

88. Li F. Survivin study: what is the next wave? J Cell Physiol 2003;197:8-29.

89. Ling X, Li F. Silencing of antiapoptotic survivin gene by multiple approaches of RNA interference technology. Biotechniques 2004;36:450-4, 6-60.

90. Tu SP, Jiang XH, Lin MC, et al. Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer. Cancer Res 2003;63:7724-32.

91. Pennati M, Binda M, Colella G, et al. Ribozyme-mediated inhibition of survivin expression increases spontaneous and drug-induced apoptosis and decreases the tumorigenic potential of human prostate cancer cells. Oncogene 2004;23:386-94.

92. Shen C, Buck A, Polat B, et al. Triplex-forming oligodeoxynucleotides targeting survivin inhibit proliferation and induce apoptosis of human lung carcinoma cells. Cancer Gene Ther 2003;10:403-10.

93. Dasgupta P, Kinkade R, Joshi B, Decook C, Haura E, Chellappan S. Nicotine inhibits apoptosis induced by chemotherapeutic drugs by up-regulating XIAP and survivin. Proc Natl Acad Sci U S A 2006;103:6332-7.

94. Li Y, Xie M, Yang J, et al. The expression of antiapoptotic protein survivin is transcriptionally upregulated by DEC1 primarily through multiple sp1 binding sites in the proximal promoter. Oncogene 2006;25:3296-306.

95. Cosgrave N, Hill AD, Young LS. Growth factor-dependent regulation of survivin by c-myc in human breast cancer. Journal of molecular endocrinology 2006;37:377-90.

96. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 2002;277:3247-57.

97. Xu Y, Fang F, Ludewig G, Jones G, Jones D. A mutation found in the promoter region of the human survivin gene is correlated to overexpression of survivin in cancer cells. DNA Cell Biol 2004;23:419-29.

98. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. Oncogene 2006;25:1125-42.

99. Deng Z, Wan M, Cao P, Rao A, Cramer SD, Sui G. Yin Yang 1 regulates the transcriptional activity of androgen receptor. Oncogene 2009.

100. Wang X, Feng Y, Xu L, et al. YY1 restrained cell senescence through repressing the transcription of p16. Biochim Biophys Acta 2008;1783:1876-83.

101. Harrison L, Blackwell K. Hypoxia and anemia: factors in decreased sensitivity to radiation therapy and chemotherapy? Oncologist 2004;9 Suppl 5:31-40.

102. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996;56:4509-15.

103. Bottaro DP, Liotta LA. Cancer: Out of air is not out of action. Nature 2003;423:593-5.

104. Hyde-DeRuyscher RP, Jennings E, Shenk T. DNA binding sites for the transcriptional activator/repressor YY1. Nucleic Acids Res 1995;23:4457-65.

105. Li F, Altieri DC. The cancer antiapoptosis mouse survivin gene: characterization of locus and transcriptional requirements of basal and cell cycle-dependent expression. Cancer Res 1999;59:3143-51.

106. Zhang N, Li X, Wu CW, et al. microRNA-7 is a novel inhibitor of YY1 contributing to colorectal tumorigenesis. Oncogene 2012.

107. de Nigris F, Zanella L, Cacciatore F, et al. YY1 overexpression is associated with poor prognosis and metastasis-free survival in patients suffering osteosarcoma. BMC Cancer 2011;11:472.

108. de Nigris F, Crudele V, Giovane A, et al. CXCR4/YY1 inhibition impairs VEGF network and angiogenesis during malignancy. Proc Natl Acad Sci U S A 2010;107:14484-9.

109. Zhang M, Yang J, Li F. Transcriptional and post-transcriptional controls of survivin in cancer cells: novel approaches for cancer treatment. J Exp Clin Cancer Res 2006;25:391-402.

110. Ai W, Liu Y, Wang TC. Yin yang 1 (YY1) represses histidine decarboxylase gene expression with SREBP-1a in part through an upstream Sp1 site. Am J Physiol Gastrointest Liver Physiol 2006;290:G1096-104.

111. Lee JS, Galvin KM, Shi Y. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. Proc Natl Acad Sci U S A 1993;90:6145-9.

112. Seto E, Lewis B, Shenk T. Interaction between transcription factors Sp1 and YY1. Nature 1993;365:462-4.

113. Mirza A, McGuirk M, Hockenberry TN, et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002;21:2613-22.

114. Tao YF, Lu J, Du XJ, et al. Survivin selective inhibitor YM155 induce apoptosis in SK-NEP-1 Wilms tumor cells. BMC Cancer 2012;12:619.

115. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.

116. Kleeff J, Michalski C, Friess H, Buchler MW. Pancreatic cancer: from bench to 5-year survival. Pancreas 2006;33:111-8.

117. Bilimoria KY, Bentrem DJ, Ko CY, Stewart AK, Winchester DP, Talamonti MS. National failure to operate on early stage pancreatic cancer. Ann Surg 2007;246:173-80.

118. Muller MW, Friess H, Koninger J, et al. Factors influencing survival after bypass procedures in patients with advanced pancreatic adenocarcinomas. Am J Surg 2008;195:221-8.

119. Ueno H, Kiyosawa K, Kaniwa N. Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy? Br J Cancer 2007;97:145-51.

120. Reni M, Cereda S, Galli L. PEFG (cisplatin, epirubicin, 5-fluorouracil, gemcitabine) for patients with advanced pancreatic cancer: the ghost regimen. Cancer Lett 2007;256:25-8.

121. Oettle H, Neuhaus P. Adjuvant therapy in pancreatic cancer: a critical appraisal. Drugs 2007;67:2293-310.

122. Olsen DR, Bruland OS, Frykholm G, Norderhaug IN. Proton therapy - a systematic review of clinical effectiveness. Radiother Oncol 2007;83:123-32.

123. Slater JD, Yonemoto LT, Mantik DW, et al. Proton radiation for treatment of cancer of the oropharynx: early experience at Loma Linda University Medical Center using a concomitant boost technique. Int J Radiat Oncol Biol Phys 2005;62:494-500.

124. Schulz-Ertner D, Tsujii H. Particle radiation therapy using proton and heavier ion beams. J Clin Oncol 2007;25:953-64.

125. DeLaney TF. Clinical proton radiation therapy research at the Francis H. Burr Proton Therapy Center. Technol Cancer Res Treat 2007;6:61-6.

126. Sarela AI, Verbeke CS, Ramsdale J, Davies CL, Markham AF, Guillou PJ. Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma. Br J Cancer 2002;86:886-92.

127. Kami K, Doi R, Koizumi M, et al. Survivin expression is a prognostic marker in pancreatic cancer patients. Surgery 2004;136:443-8.

128. Kim KW, Mutter RW, Willey CD, et al. Inhibition of survivin and aurora B kinase sensitizes mesothelioma cells by enhancing mitotic arrests. Int J Radiat Oncol Biol Phys 2007;67:1519-25.

129. Asanuma K, Kobayashi D, Furuya D, Tsuji N, Yagihashi A, Watanabe N. A role for survivin in radioresistance of pancreatic cancer cells. Jpn J Cancer Res 2002;93:1057-62.

130. Pennati M, Binda M, Colella G, et al. Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression. J Invest Dermatol 2003;120:648-54.

131. Kami K, Doi R, Koizumi M, et al. Downregulation of survivin by siRNA diminishes radioresistance of pancreatic cancer cells. Surgery 2005;138:299-305.

132. Lee JU, Hosotani R, Wada M, et al. Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. Eur J Cancer 1999;35:1374-80.

133. Li F, Ackermann EJ, Bennett CF, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat Cell Biol 1999;1:461-6.

134. O'Connor DS, Wall NR, Porter AC, Altieri DC. A p34(cdc2) survival checkpoint in cancer. Cancer Cell 2002;2:43-54.

135. Dohi T, Xia F, Altieri DC. Compartmentalized phosphorylation of IAP by protein kinase A regulates cytoprotection. Mol Cell 2007;27:17-28.

136. Moertel CG, Childs DS, Jr., Reitemeier RJ, Colby MY, Jr., Holbrook MA. Combined 5-fluorouracil and supervoltage radiation therapy of locally unresectable gastrointestinal cancer. Lancet 1969;2:865-7.

137. Park JK, Ryu JK, Lee JK, et al. Gemcitabine chemotherapy versus 5-fluorouracil-based concurrent chemoradiotherapy in locally advanced unresectable pancreatic cancer. Pancreas 2006;33:397-402.

138. Burris HA, 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997;15:2403-13.

139. Trofimov A, Nguyen PL, Coen JJ, et al. Radiotherapy treatment of early-stage prostate cancer with IMRT and protons: a treatment planning comparison. Int J Radiat Oncol Biol Phys 2007;69:444-53.

140. Giagkousiklidis S, Vellanki SH, Debatin KM, Fulda S. Sensitization of pancreatic carcinoma cells for gamma-irradiation-induced apoptosis by XIAP inhibition. Oncogene 2007;26:7006-16.

141. Karikari CA, Roy I, Tryggestad E, et al. Targeting the apoptotic machinery in pancreatic cancers using small-molecule antagonists of the X-linked inhibitor of apoptosis protein. Mol Cancer Ther 2007;6:957-66.

142. Comai L. The advantages and disadvantages of being polyploid. Nat Rev Genet 2005;6:836-46.

143. Storchova Z, Pellman D. From polyploidy to aneuploidy, genome instability and cancer. Nat Rev Mol Cell Biol 2004;5:45-54.

144. Ganem NJ, Storchova Z, Pellman D. Tetraploidy, aneuploidy and cancer. Curr Opin Genet Dev 2007;17:157-62.

145. Mufti AR, Burstein E, Duckett CS. XIAP: cell death regulation meets copper homeostasis. Arch Biochem Biophys 2007;463:168-74.

146. Lee KB, Kim KR, Huh TL, Lee YM. Proton induces apoptosis of hypoxic tumor cells by the p53-dependent and p38/JNK MAPK signaling pathways. Int J Oncol 2008;33:1247-56.

147. Thayaparasingham B, Kunz A, Peters N, Kulms D. Sensitization of melanoma cells to TRAIL by UVB-induced and NF-kappaB-mediated downregulation of xIAP. Oncogene 2009;28:345-62.

148. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.

149. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.

150. Mees C, Nemunaitis J, Senzer N. Transcription factors: their potential as targets for an individualized therapeutic approach to cancer. Cancer Gene Ther 2009;16:103-12.

151. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155-62.

152. Baritaki S, Huerta-Yepez S, Sakai T, Spandidos DA, Bonavida B. Chemotherapeutic drugs sensitize cancer cells to TRAIL-mediated apoptosis: up-regulation of DR5 and inhibition of Yin Yang 1. Mol Cancer Ther 2007;6:1387-99.

153. Huerta-Yepez S, Vega M, Escoto-Chavez SE, et al. Nitric oxide sensitizes tumor cells to TRAIL-induced apoptosis via inhibition of the DR5 transcription repressor Yin Yang 1. Nitric Oxide 2009;20:39-52.

154. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Current Opinion in Cell Biology 2005;17:548-58.

155. Kurrey NK, K A, Bapat SA. Snail and Slug are major determinants of ovarian cancer invasiveness at the transcription level. Gynecologic Oncology 2005;97:155-65.

156. Medici D, Hay ED, Olsen BR. Snail and Slug Promote Epithelial-Mesenchymal Transition through {beta}-Catenin-T-Cell Factor-4-dependent Expression of Transforming Growth Factor-{beta}3. Mol Biol Cell 2008;19:4875-87.

157. Yang J, Mani SA, Donaher JL, et al. Twist, a Master Regulator of Morphogenesis, Plays an Essential Role in Tumor Metastasis. Cell 2004;117:927-39.

158. Khan S, Aspe JR, Asumen MG, et al. Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential. Br J Cancer 2009;100:1073-86.

159. Yie SM, Lou B, Ye SR, et al. Clinical significance of detecting survivin-expressing circulating cancer cells in patients with non-small cell lung cancer. Lung Cancer 2008.

160. Yang D, Schneider S, Azuma M, et al. Gene expression levels of epidermal growth factor receptor, survivin, and vascular endothelial growth factor as molecular markers of lymph node involvement in patients with locally advanced rectal cancer. Clin Colorectal Cancer 2006;6:305-11.

161. Ikehara M, Oshita F, Kameda Y, et al. Expression of survivin correlated with vessel invasion is a marker of poor prognosis in small adenocarcinoma of the lung. Oncol Rep 2002;9:835-8.

162. Lo Muzio L, Staibano S, Pannone G, et al. Expression of the apoptosis inhibitor survivin in aggressive squamous cell carcinoma. Exp Mol Pathol 2001;70:249-54.

163. Ulf Henning Beier TG. Implications of galactocerebrosidase and galactosylcerebroside metabolism in cancer cells. International Journal of Cancer 2005;115:6-10.

164. Wang CC, Tsai MF, Hong TM, et al. The transcriptional factor YY1 upregulates the novel invasion suppressor HLJ1 expression and inhibits cancer cell invasion. Oncogene 2005;24:4081-93.

165. Wang CC, Tsai MF, Dai TH, et al. Synergistic activation of the tumor suppressor, HLJ1, by the transcription factors YY1 and activator protein 1. Cancer Res 2007;67:4816-26.

166. Palmer MB, Majumder P, Cooper JC, Yoon H, Wade PA, Boss JM. Yin yang 1 regulates the expression of snail through a distal enhancer. Mol Cancer Res 2009;7:221-9.

167. Baumeister P, Luo S, Skarnes WC, et al. Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. Mol Cell Biol 2005;25:4529-40.

168. Gronroos E, Terentiev AA, Punga T, Ericsson J. YY1 inhibits the activation of the p53 tumor suppressor in response to genotoxic stress. Proc Natl Acad Sci U S A 2004;101:12165-70.

169. Wan M, Huang W, Kute TE, et al. Yin Yang 1 plays an essential role in breast cancer and negatively regulates p27. Am J Pathol 2012;180:2120-33.