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A pharmacogenomic approach to treating brain and pancreatic cancers using capecitabine chemoradiotherapy.

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A PHARMACOGENOMIC APPROACH TO TREATING BRAIN AND PANCREATIC CANCERS USING CAPECITABINE CHEMORADIOTHERAPY

by

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A DISSERTATION

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in partial fulfillment of the requirements for the degree of
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ABSTRACT OF DISSERTATION
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Cancer has been afflicting populations worldwide throughout recorded history. Despite making significant progress in the treatment of cancer, the prognosis for most cancer types remains grim. Thus, novel approaches for treating this disease are still needed. Pharmacogenomics, a branch of pharmacology particularly relevant to the cancer research field, is the study of how variations in the genome affect response to pharmaceutical agents and influence biological pathways and processes, including the pharmacokinetics, pharmacodynamics, efficacy, and toxicity of a drug. The observations detailed in this dissertation suggest that, by utilizing a pharmacogenomic approach, it is possible to design novel therapies or regimens to ultimately treat patients with highly lethal cancers, specifically glioblastoma multiforme (GBM) and pancreatic andenocarcinoma (PAC). We achieved this objective through validation of gene expression quantitation methods and by focusing on drug-metabolizing enzymes, such as thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase, which have been shown by us and others to affect therapeutic response, efficacy, and toxicity of 5-fluorouracil-based agents such as capecitabine (CAPE; a novel, orally administered 5-fluorouracil prodrug). Molecular analysis demonstrating elevated TP levels in GBM, as well as the up-regulation of TP with radiotherapy in glioma xenografts (possibly as a result of cytokine mediation), provided the ra-

tionale to examine CAPE chemoradiotherapy for GBM. Analyses of PAC, which has a molecular profile similar to that of GBM, also suggested CAPE use; however, radiotherapy did not increase TP, as evidenced by studies in xenografts and clinical samples. Nevertheless, CAPE-radiotherapy demonstrated synergistic antitumor efficacy in PAC xenografts. These results provided the foundation for the design and realization of 2 Phase-I clinical trials conducted at our institution, which demonstrated low toxicity of CAPE-radiotherapy in both PAC and GBM patients. Additionally, in GBM patients, one complete remission was documented. These studies will be fundamental in establishing the rationale to further develop clinical studies and protocols. In summary, the observations detailed herein provide compelling evidence that utilizing a pharmacogenomic approach to design CAPE chemoradiotherapy treatment regimens for PAC and GBM patients is a rational choice and provides a feasible and a potentially effective method for improving response to chemotherapy, in terms of both reduced toxicity and enhanced efficacy.

DEDICATION

This dissertation is dedicated to my parents, Carmelo Enrique and Esmeralda Olier de Blanquicett, for their unconditional and perpetual support. I would also like to make a dedication to my family, including my "abuela" Juanita and my "tia" Berta, as well as the memory of Mrs. Martha Sanders Falls. Moreover, through this body of work, I would like to encourage my niece, Halley-Camille, to accomplish any goal she desires in life.

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The highest reward for a man's toil is not what he gets for it but what he becomes by it.

–John Ruskin

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LIST OF ABBREVIATIONS

A_{260}	Absorbance of 260 nm
β-2m	β-2 microglobulin
β-actin	β-smooth muscle actin
BID	Twice a day
BBB	Blood-brain barrier
CAPE	Capecitabine
COX-2	Cyclooxygenase-2
CYC	Cyclophylin
dATP	2'-deoxyadenine triphosphate
dCTP	2'-deoxycytidine triphosphate
dGTP	2'-deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DPD	Dihydropyrimidine Dehydrogenase
dUTP	2'-deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FdUTP	5'-fluoro-2'-deoxyuridine triphosphate
5'-FU	5'-fluorouracil
FUTP	5'-fluorouridine triphosphate

G6PDH	glyceraldehyde 6-phosphate Dehydrogenase
GAPDH	glyceraldehyde 3-phosphate Dehydrogenase
GBM	glioblastoma multiforme
GUS	β–glucuronidase
HKG	Housekeeping gene
HPRT	Hypoxanthine ribosyl transferase
huPO	Human Acidic ribosomal protein
huTfR	Human Transferin receptor
IFN-γ	interferon gamma
IL-10	interleukin 10
IL-1α	interleukin 1 alpha
IL-1β	interleukin 1beta
mRNA	messenger ribonucleic acid
OPRT	Orotate phosphoribosyl transferase
PAC	Pancreatic adenocarcinoma
PBS	Phosphate buffered saline
PGK	Phosphoglycerokinase
Q-RT-PCR	quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
S9	S9 Ribosomal subunit
TBP	TATA binding protein

TNF	Tumor necrosis factor
TP	Thymidine Phosphorylase
Tris	Tris(hydroxymethyl)aminomethane
TS	Thymidylate synthase
UTR	Untranslated region
XRT	External Beam Radiotherapy

INTRODUCTION

Pharmacogenomics and Its Role in Cancer

Despite decades of intensive research and phenomenal advances in cancer therapeutics, cancer remains the second leading cause of death in the United States and in most developed countries (Tiwari et al., 2004). Applying pharmacogenomics in the cancer research field may be of great benefit.

The term *pharmacogenomics* was introduced in 1995 to describe the branch of pharmacology dedicated to the study of how pharmaceutical agents interact with the genome to influence biological pathways and processes, including pharmacokinetics, pharmacodynamics, efficacy, and toxicity within a host. Pharmacogenomics is closely related to pharmacogenetics (the study of the heritable response to pharmaceutical agents, or gene–drug interactions); often, the distinction between the two terms is unclear, resulting in their being used interchangeably. Since the coinage of the term, the number of sources related to pharmacogenomics has grown exponentially and continues to expand, indicating that this is an area that is given and will be given much attention in the years to come. More importantly, this discipline may be applied to diseases where success in treatment has not yet been consistently evident, such as cancer.

It is well established that cancer treatments oftentimes fail and have serious toxic side effects. This failure and/or the resultant serious side effects may be caused by the fact that tumors often inherently contain or acquire a mutation that makes them resistant to chemotherapy. At times, it may be the host who has a genetic variance in the genes

coding for drug-metabolism enzymes, drug transporters, or drug modifying proteins, ultimately affecting the response to treatment.

By means of drug-level monitoring, the goal of clinical pharmacology for the past 5 decades has been to individualize the dosage of many drugs, particularly those with low therapeutic indices (such as anticancer drugs), to reduce toxicity. Genetic variances in drug-catabolizing or -clearing enzymes may also render a host susceptible to serious adverse events, particularly with drugs having narrow therapeutic indices or "windows" (e.g., cancer chemotherapeutics). Pharmacogenomic strategies have great utility in potentially identifying both patients who may respond to treatment and those who are at risk for serious drug reactions; additionally, pharmacogenomics may also have a role in the design of novel treatment regimens. On the basis of gene expression differences within each subject, pharmacogenomics can be applied in an attempt to anticipate and minimize each subject's risk of experiencing an adverse drug reaction. Unfortunately, serious adverse events, including therapeutic failure, are very common in the clinic. In support, a large prospective study showed that, during a single year, more than 2 million hospitalized patients experience serious adverse drug reactions, and more than 100,000 fatalities occur as a result—ranking adverse drug reactions as the fifth leading cause of death in the United States (Lazarou, Pomeranz, & Corey, 1998). Furthermore, adverse reactions caused specifically by cancer chemotherapy are estimated to increase the overall hospital costs by 1.9% and drug costs by 15% (Wiffen, 2002).

Determination of the complete molecular basis for both host toxicity and tumor response not only requires a solid understanding of the metabolism of the drug, its therapeutic mechanism of action and response, and the gene(s) coding for the enzyme(s) involved in drug metabolism but also requires an understanding of the subtle individual dif-

ferences within a host or a tumor type that may alter all of these factors. Ideally, the best and most efficacious drug for a particular patient's condition or cancer type could be chosen (tailored treatment) on the basis of the host's genetic makeup or a tumor's molecular profile without subjecting that person to serious adverse events.

The combination of a high therapeutic failure rate, a large number of serious adverse events–particularly with cancer chemotherapy–and the increasing cost of health care requires rational, promising, and novel approaches or strategies that may address these concerns. Applying pharmacogenomics to improve cancer therapy may be one such relatively novel approach. Studies from members of our laboratory and from other researchers have demonstrated that, by determining the level of important drug-metabolizing enzymes inside the tumor, it is possible to predict how that tumor will respond to a particular drug (Diasio & Johnson, 1999; Salonga et al., 2000). In addition to identifying cancer patients who would respond to a particular chemotherapy drug, as mentioned, pharmacogenomic studies may allow researchers to design and combine potentially synergistic therapies.

Difficulties of Pharmacogenomic Strategies

Nebert and Vesell (2004) contend that it is very difficult to define a phenotype and a genotype "unequivocally." The magnitude of difficulty in unequivocally identifying a particular phenotype or genotype is becoming increasingly apparent and exists for a number of reasons, including overlaps in drug substrate specificity; drug-metabolizing enzymes, receptors, and ion channels; genetic heterogeneity of drug transporters, transcription factors, and receptors; and drug interactions with other drugs (or with the environment). The multifactorial components influencing genotype and phenotype within a host add an entire level of complexity and difficulty in unequivocally predicting

host add an entire level of complexity and difficulty in unequivocally predicting response or toxicity. Thus, individual response to therapeutics is highly variable (Nebert & Vesell). This variability suggests that there may exist countless phenotypic exceptions and subtle variations among individuals that could prevent the safe clinical application of a specific set of results to individual patients.

Recent Successful Applications

Nevertheless, despite the potential difficulty of using general genotypic characteristics as response indicators, recent studies have demonstrated the potential success of applied pharmacogenomics. A particular example of how pharmacogenomics has been successfully applied was recently reported in patients being treated with the anticancer agent, gefitinib (Lynch et al., 2004; Paez et al., 2004). Less than 10% of Caucasian persons with non-small-cell lung cancer but approximately 25% of Japanese individuals with this cancer respond favorably to gefitinib (Iressa) if they have particular mutations in the tyrosine kinase domain of the epidermal growth factor receptor gene of their tumor. This finding suggests that determination of intratumoral epidermal growth factor receptor gene status can be a means of predicting response to gefitinib. Dramatic therapeutic responses (96%) seen with Gleevec have also stirred much enthusiasm in the cancer pharmacogenomics field within the last few years (Kantarjian et al., 2004). These results have demonstrated the potential benefits of using gene expression status or tumor type to stratify patients who may be good responders to a specific anticancer agent. Equally important to predicting response is the prediction of toxicity, which has been successfully demonstrated in the last decade. One particular example illustrating the concept involves the anticipation of life-threatening toxicity by determining dihydropyrimidine dehydrogenase

(DPD) enzyme levels (Diasio, Beavers, & Carpenter, 1988), as briefly mentioned previously. These select examples demonstrate that a pharmacogenomic approach is feasible, has useful applications, and already has (and may have further) significant potential, as evidenced recently and despite the difficulties faced in using this approach.

Overall Goal

It is possible that almost any therapeutic compound may have pharmacogenomic applications, provided that careful gene expression validation studies are performed, thorough drug metabolism pathways are examined, and studies on the regulation of drug-metabolizing genes are conducted. The studies described herein involve attempts to apply pharmacogenomics in the cancer treatment area by using the chemotherapy agent, capecitabine (CAPE), for tumors exhibiting a characteristic molecular profile indicative of response to this drug. More specifically, the objective of the studies detailed in this dissertation is to utilize a broader approach to pharmacogenomics to design novel therapies or therapeutic regimens that would ultimately be used to treat patients with highly lethal cancers such as glioblastoma multiforme (GBM) and pancreatic andenocarcinoma (PAC). This goal can be achieved through carefully validating gene expression quantitation methods and focusing on particular drug-metabolizing enzymes such as thymidine phosphorylase (TP) and DPD, which have been shown by members of our laboratory and by other researchers to affect the therapeutic response, efficacy, and toxicity of 5-fluorouracil (5-FU)-based agents (e.g., CAPE)

CAPE Metabolism

CAPE (N^4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) is an oral prodrug of 5-FU (Bajetta, Carnaghi, Somma, & Stampino, 1996). After administration and as illustrated in Figure 1, CAPE reaches the liver through portal circulation, where it is metabolized to 5'-deoxy-5-fluorocytidine by carboyxlesterase (Wang, Yung, Royce, Schomer, & Theriault, 2001). Cytidine deaminase, which is widely distributed throughout the body converts 5'-deoxy-5-fluorocytidine into 5'-deoxy-5-fluorouridine. Finally, 5'-deoxy-5-fluorouridine is hydrolyzed into 5-FU through the activation by the rate-limiting enzyme, TP (Bajetta et al.; Budman et al., 1998; Sawada et al., 1998). Once CAPE is intracellularly converted into 5-FU by TP, the biochemical mechanisms responsible for the antitumor effects require anabolism into specific 5-FU cytotoxic nucleotides. This anabolism is now known to be accomplished by three pathways: (a) incorporation of 5'-fluorouridine triphosphate into RNA, which can interfere with transcription and translation; (b) incorporation of 5'-fluoro-2'-deoxyuridine triphosphate into DNA, which can result in strand breakage; or (c) inhibition of thymidylate synthase through the formation of a ternary complex consisting of thymidylate synthase, the folate cofactor 5,10 methylene tetrahydrofolate, and 5'-fluoro-2'-deoxyuridine monophosphate. Inhibition of thymidylate synthase, thought to be the primary mechanism of action, results in the depletion of cellular thymidylate pools, thus inhibiting DNA replication and cell division (Grem, 1996). Clearance or catabolism of 5-FU is performed mainly by DPD. It is now known that 80-90% of an administered dose of 5-FU is inactivated by DPD, the initial rate-limiting enzyme in pyrimidine catabolism that reduces 5-FU into dihydrofluorouracil, an inactive metabolite (Diasio & Johnson, 1999; Heggie, Sommadossi, Cross, Huster, & Diasio, 1987). Thus, this enzymatic step (which is essentially

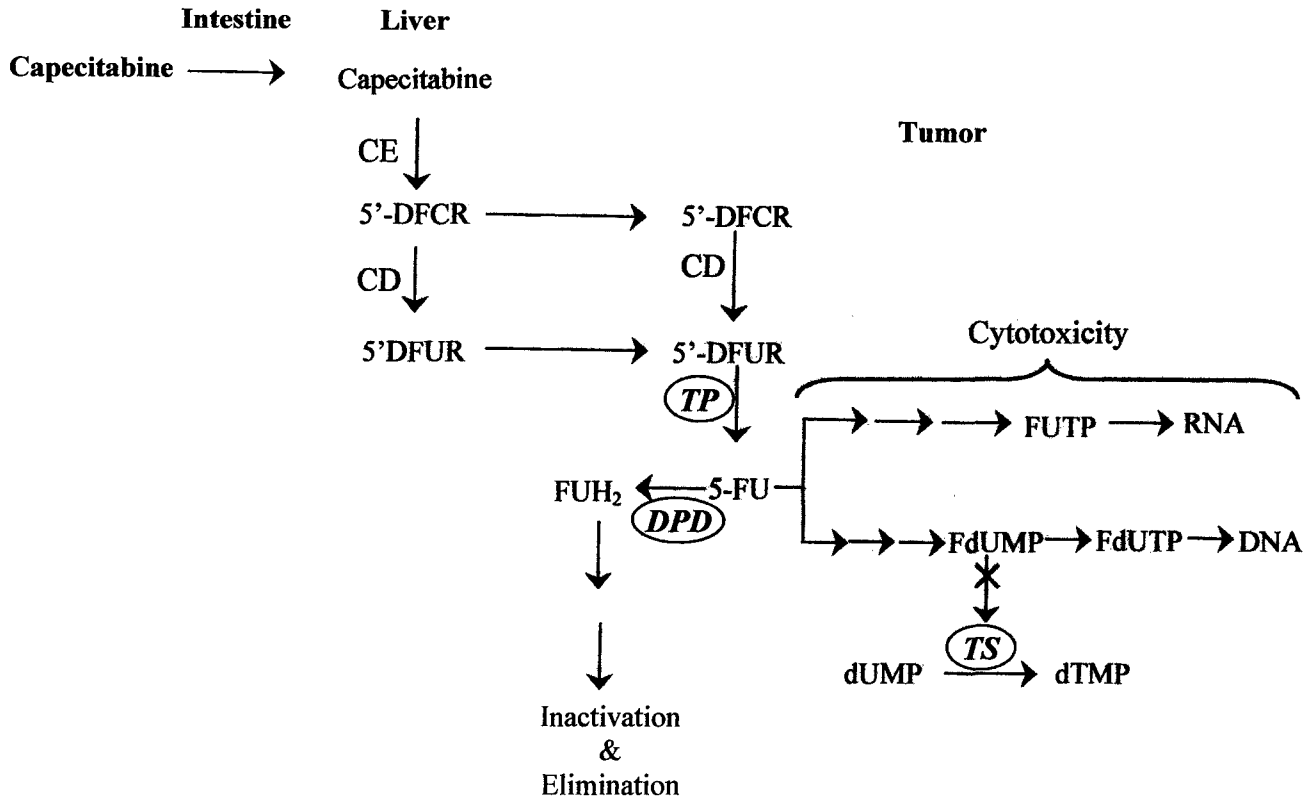

Figure 1. Metabolic pathway of capecitabine. 5'-DFCR = 5'-deoxy-5-fluorocytidine; CD = Cytidine deaminase; 5'-DFUR = 5'-deoxy-5-fluorouridine; dThdPase or TP = thymidine phosphorylase; FdUMP = 5-fluoro-2'-deoxyuridine monophosphate; FUTP = 5-fluorouridine triphosphate; UTP = uridine triphosphate; DPD = dihydropyrimidine dehydrogenase.

physiologically unidirectional) is important in the overall regulation of 5-FU efficacy. Pharmacokinetic studies have shown that decreased DPD activity (DPD deficiency) can result in decreased 5-FU clearance, increased half-life, and a concurrent increase in anabolism, ultimately leading to increased toxicity (Diasio et al., 1988). As a result, a delicate balance exists between the enzymatic activation of 5-FU by TP (anabolism) and its catabolic elimination (by DPD).

Several recent studies have shown that the level of DPD inside the tumor can have a significant effect on the likelihood that the tumor will respond to 5-FU (Beck et al., 1994; Etienne et al., 1995; Jiang, Lu, He, & Diasio, 1997). Those tumors with high levels of DPD can efficiently catabolize 5-FU, leaving minimal 5-FU to be anabolized and thus providing a possible mechanism of 5-FU resistance. This finding has provided the stimulus for the development of new drugs that specifically inhibit DPD, such that concomitant administration of 5-FU may result in improved antitumor efficacy (Cao, Rustum, & Spector, 1994). However, DPD is only one of the components associated with efficacy and toxicity of CAPE or 5-FU. Because TP is responsible for CAPE activation, its levels can also be used as an indicator of response. Therefore, in conjunction, both TP and DPD (expressed as a ratio of TP/DPD) levels may be potentially used either to stratify patients who could respond to CAPE or to examine tumor types that could respond to CAPE.

GBM

GBM is one such tumor that may be treated with CAPE. Despite >50 years of research, GBM remains the most common and malignant type of brain tumor and is associated with a median survival of <1 year in most patients (Macarthur & Buxton, 2001; Prados & Levin, 2000). The current management of most glial tumors includes surgical de-

bulking, with most studies supporting the use of external beam radiotherapy (XRT) (Gilbert, 2005). Until recently, chemotherapy had not generally demonstrated survival benefit in this disease, and severe dose-related toxicity of frequently used nitrosourea agents limited the usefulness of these compounds (Subach et al., 1999). However, preliminary molecular analysis of GBM and normal brain tissues by members of our laboratory suggested that CAPE would be selectively activated in GBM, compared to normal brain tissue. Furthermore, subsequent studies showed that the use of XRT up-regulates TP, meaning that XRT before the administration of CAPE may have a significant tumor-associated, additive or synergistic effect on the antitumor efficacy of this compound. Because XRT remains a current standard of care for the treatment of GBM, the addition of CAPE represents a rational and potentially effective chemoradiation treatment. The hypothesis that CAPE may be used to treat brain malignancies is highly supported by recent case studies documenting that brain metastases originating in breast cancer can respond completely after CAPE administration (Siegelmann-Danieli, Stein, & Bar-Ziv, 2003) and partially, after 2 months of CAPE treatment (Wang et al., 2001).

PAC

Pancreatic cancer is another highly lethal cancer that has one of the poorest prognoses of all gastrointestinal malignancies. Pancreatic cancer remains the fourth leading cause of cancer-related deaths in Western industrialized countries (Czito, Willet, & Clark, 2000). The incidence of PAC, the most common type of pancreatic cancer, has been increasing throughout the past decade (Shi, Friess, Kleeff, Ozawa, & Buchler, 2001). PAC is characterized by not only poor prognosis (3 to 6 months without treatment) but also by late diagnosis and lack of response to conventional therapies (high chemoresistance;

Ding, Tong, & Adrian, 2001). Conventional therapy for advanced disease had relied on palliative 5-FU-based chemotherapy but with unsatisfactory results. Combinations of gemcitabine (the new standard of treatment for PAC) with antitumor drugs such as 5-FU and docetaxel have yielded promising results in Phase II trials; however, none of these combinations has yet reached the level of an evidence-based standard treatment (Heinemann, 2002). Consequently, new targets and/or chemotherapeutic strategies also need to be identified for pancreatic cancer treatment. By examining PAC's molecular profile, it may be possible to achieve the goal of designing novel and promising treatment regimens for this disease.

Our preliminary data suggested that TP, as is the case in GBM, is also up-regulated in carcinomatous pancreatic tissue relative to normal pancreatic tissue. Because XRT and chemotherapy (5-FU and now gemcitabine) are considered the standard treatment for locally advanced disease and because XRT has been shown to increase TP levels in colon cancer (another common gastrointestinal malignancy) xenografts, the combination of CAPE-XRT would also be a rational choice for PAC, especially for patients with advanced disease where resection is not feasible. In addition to TP, our data also suggest that cyclooxygenase-2 (COX-2) is overexpressed in pancreatic carcinomatous tissues. This finding is in agreement with results found by other investigators using immunocytochemical, reverse transcription-polymerase chain reaction, and Western blotting; these results demonstrated that COX-2 expression is higher in human pancreatic cancer cell lines, as well as in human pancreatic cancer tissue, than in normal ductal cells or normal pancreas specimens. Thus, combination strategies involving COX-2 inhibitors (e.g., celecoxib) in addition to CAPE-XRT may add potential utility to the treatment of PAC.

Central Hypothesis

The central hypothesis of this dissertation is that a pharmacogenomic approach can be used to design novel therapies or therapeutic regimens to treat cancers such as GBM and PAC, highly lethal cancers with grim prognoses. The molecular profile exhibited by GBM and PAC suggests that a CAPE chemotherapy regimen is a rational and potentially efficacious choice. A long-term objective is that these studies will be fundamental in establishing a rationale to further develop clinical studies/protocols based on gene expression differences. Our broad approach may also be used to treat other cancer types, particularly those where chemotherapeutics have not yet been optimized.

Specific Aims

To study pharmacogenomics, accurate gene expression quantification becomes crucial, particularly when cancer chemotherapy is involved (narrow therapeutic windows). The introduction of real time-quantitative-polymerase chain reaction (RT-Q-PCR) has allowed gene expression quantification from samples that were previously difficult to quantify because they were limited or in exiguous amounts (such as needle biopsy samples). In the past, we have developed and validated a high-throughput RT-Q-PCR method that is capable of reliable and accurate determination of messenger RNA (mRNA) levels in biopsy-sized tissue samples and other limited samples. This RT-Q-PCR method has been validated in our laboratory through correlation with enzyme activity, Northern blot analysis, immunocytochemistry, and enzyme-linked immunosorbent assays (Johnson, Wang, Smith, Heslin, & Diasio, 2000). In the context of cancer, differences in gene expression levels have been associated with tumor metastasis, tumor progression, and systemic toxicity (Bowen et al., 2000; Jackson & Puisieux, 2000; Johnson et al., 1999; We-

ber & Ashkar, 2000). Furthermore, because subtle changes in gene expression may potentially translate to dramatic phenotypic changes such as altered response to or unexpected toxicity from cancer chemotherapy, precision in gene expression is essential (recall that the boundary between efficacy and toxicity is not very distinct for cancer chemotherapeutics).

One critical requirement in the accurate quantitation of gene expression is the availability of a ubiquitously expressed housekeeping gene (HKG) to normalize the amount of total RNA in each sample. The potential variation in expression among different HKGs, especially in carcinomatous (hypermetabolic) tissues, could affect pharmacogenomic-type studies and should therefore be evaluated. If variation in expression among HKGs is observed, an optimal HKG (one that does not demonstrate significant variability across tissue types) can be chosen as adequate for conducting pharmacogenomic studies and as particularly applicable to cancer research. Because of its sensitivity and quantitative capacity, RT-Q-PCR proved especially useful in determining (a) whether variation in expression among HKGs occurs in normal versus carcinomatous tissues and, if so, (b) whether an adequate HKG could be identified to conduct pharmacogenomic studies. These questions led us to our first specific aim.

Specific Aim 1

We aimed to identify in Specific Aim 1 the identification of an HKG that does not demonstrate variation in expression between normal and carcinomatous tissues.

Examination of 15 HKGs led to our selection of the S9 ribosomal HKG because of its minimal (less than 2-fold) variation in expression across various tissues (see article 1 in this dissertation). However, whether one HKG could generalize to other tissue types

was not known. Nevertheless, the approach used in the HKG studies could be employed to determine HKG variation among other tissues of interest before conducting pharmacogenomic studies involving cancer and the use of cancer chemotherapeutics. Using a similar methodology, we also examined HKG variation in normal brain and GBM tissue samples. As shown in Figure 2, results of this evaluation indicated that S9 could also be used to normalize nucleic acid amounts in GBM tissue. Consequently, gene expression analyses of GBM could now be conducted with greater accuracy.

Although we suggest the design of chemotherapeutic regimens on the basis of gene expression profiling of a tumor type, it is also of interest to alter a specific tumor's (or patient's) gene expression profile to make that tumor (which may have been previously resistant) susceptible to treatment with chemotherapy. GBM is an example of a tumor type that is resistant to chemotherapy. Determining the molecular profile of GBM and potentially altering that gene expression profile could represent a potential way of making those tumors sensitive (or more sensitive) to chemotherapy.

Specific Aim 2

The aim of Specific Aim 2 involved the examination of the molecular profile of GBM and normal brain tissues and determination of the effect of XRT on TP and DPD in a glioma mouse model of brain metastasis.

Because results after completion of this aim demonstrated that TP was elevated in GBM and, interestingly, up-regulated by XRT in glioma xenografts, it became evident that the regulation of this enzyme warranted further investigation. Although these results further support the rationale for evaluating CAPE-XRT in gliomas, it is not clear how XRT induces TP. Previous studies have shown cytokines to increase immediately after

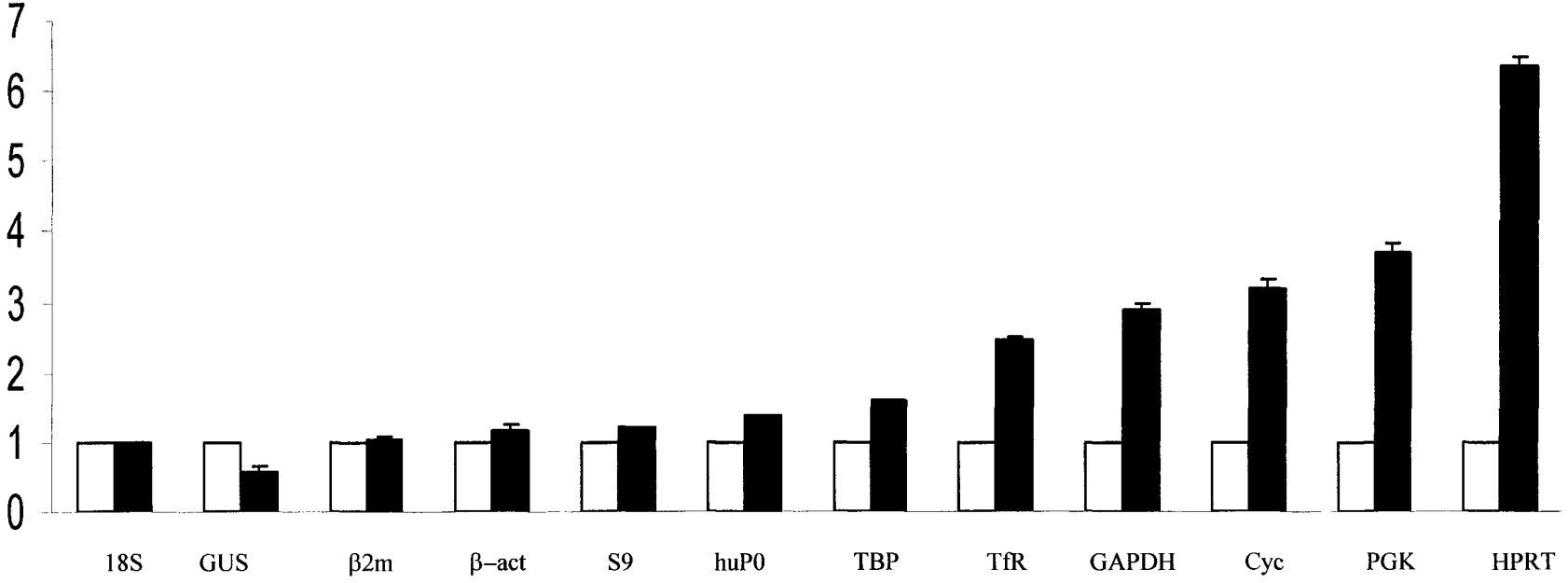

Figure 2. Housekeeping gene expression in glioblastoma multiforme. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Cyc = cyclophilin; PGK = 3-phosphoglycerokinase; HPRT = hypoxanthine-guanine phosphoribosyl transferase; TBP = TATA-binding protein; TfR = transferring receptor; GUS = β-glucuronidase; huPO = human acidic ribosomal protein.

XRT. Our observation suggesting that shielded, contralateral tumors also demonstrate increased TP levels after XRT, as well as the fact that there is a delay before maximal levels of TP are reached in those tumors, indicates to us the possibility that soluble cell factors (such as cytokines) could be involved in the communication and remarkable TP up-regulation in irradiated and shielded tumors. Therefore, cytokines were examined as being potentially implicated in the regulation of TP expression.

Specific Aim 3

We proposed in Specific Aim 3 to elucidate the mechanisms regulating TP expression by determining the possible cause for TP's increase after XRT of glioma xenografts.

These studies suggest a possible mechanism for our reported XRT induction of TP expression. XRT modulation of TP may be mediated through cytokine induction and stabilization of TP mRNA. Furthermore, protein binding at the NF-kB, AP2, and Sp1 sites of the TP promoter is also demonstrated, suggesting that these transcription factors are potentially involved in TP transcriptional activation. Additionally, results also suggest that the 3' untranslated region of the TP gene may also be involved as a post-transcriptional mechanism for up-regulation. Subsequent studies will be useful in thoroughly examining the 3' end and the 3' untranslated region of the TP gene to determine stabilizing factors binding to that region. Ultimately, knowledge of the mechanisms responsible for TP regulation could provide a means for improving the efficacy of CAPE.

The exciting possibility of altering tumor gene expression to potentially enhance chemotherapy (CAPE) efficacy in a highly lethal tumor type resistant to therapy is worthy of examination in other lethal and treatment-resistant cancers besides GBM. Because

highly-lethal cancers have such few chemotherapeutic options and because survival time is short after a diagnosis of these cancers, potentially efficacious treatment strategies observed in one cancer type should be (and are commonly) examined in other cancer types. The hypothesis that CAPE chemoradiotherapy could be effective in tumors overexpressing TP, including in tumors outside the irradiated field, not only requires confirmation but is especially relevant to PAC, in which metastatic disease is the primary source of morbidity and in which preliminary studies also suggested TP overexpression. Therefore, we established a fourth aim which consisted of performing a molecular analysis of PAC and examining the antitumor efficacy of CAPE-XRT in a PAC metastatic model.

Specific Aim 4

We aimed in Specific Aim 4 to determine the gene expression profile of PAC and to examine CAPE chemoradiotherapy efficacy in a pancreatic cancer model of metastasis.

These studies suggest that CAPE-XRT is a synergistic antitumor combination that can be improved with COX-2 inhibitors; interestingly, this combination is also effective in tumors outside the irradiated field via abscopal effects (effects of localized XRT on distant tumors outside the irradiated field). The promising results obtained from these preclinical studies warrant examination or translation into the clinic. These results led to the pursuit of our fifth and final specific aim.

Specific Aim 5

We proposed in Specific Aim 5 to present clinical evidence on the feasibility of and the rationale for translating promising PAC preclinical studies into the clinic; accord-

ingly, we established a Phase I clinical trial to examine CAPE chemoradiotherapy for PAC patients.

This study was initiated to provide preliminary results on the tolerability of CAPE chemoradiation in patients with locally advanced, unresectable pancreatic cancer. The results of this particular trial suggest that CAPE chemoradiotherapy is tolerable, with low toxicity, and is potentially effective in patients with locally advanced pancreatic cancer. Furthermore, the examination of the determinants of response to CAPE (TP and DPD and their modulation by XRT), demonstrated high concordance with our preclinical studies. A similar evaluation in a separate trial for GBM patients also demonstrated low toxicity of a CAPE-XRT dual-modality treatment combination. Additionally (although not statistically significantly), one complete remission was reported in that trial.

Significance of the Study

According to the American Cancer Society, cancer is the second leading cause of death in the United States. Cancers such as those of the brain and pancreas, have grim prognoses, partly as a result of the propensity for metastatic disease and the limited treatment options available. Optimization of chemotherapy will be necessary to improve outcome and median survival in these types of cancers. Using a pharmacogenomic approach, the gene expression profile of these cancers can be characterized and ultimately exploited by targeting chemotherapy or developing regimens that may be specific to the tumor's characteristic molecular profile. Understanding the genomic factors affecting efficacy and toxicity of a drug (pharmacogenomics) can become the paradigm of future treatment approaches.

This dissertation establishes the rationale for a CAPE chemoradiotherapy regimen to treat PAC and GBM; the rationale is based on the molecular profile exhibited in those tumor types, as well as on the results of our preclinical and clinical studies, demonstrating the synergistic antitumor efficacy of this combination and suggesting low toxicity (further illustrating the feasibility of this regimen), respectively. Additionally, this oeuvre will allude to mechanisms involved in the regulation of the CAPE-metabolizing enzyme, TP. Knowledge of the regulation of TP may allow a better tailoring, optimizing, and understanding of the efficacy of the drug CAPE, whether given alone or in combination. A better understanding of the factors affecting (or improving) response to CAPE should ultimately, improve outcome, not only in malignancies where this drug is currently used but in those where CAPE is proposed to be efficacious, as well.

HOUSEKEEPING GENE VARIABILITY IN NORMAL AND CARCINOMATOUS COLORECTAL AND LIVER TISSUES: APPLICATIONS IN PHARMACOGENOMIC GENE EXPRESSION STUDIES

by

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Analytical Biochemistry, 303, 209-214

Format adapted and errata corrected for dissertation

Introduction

Early molecular studies examining cancer susceptibility and progression have now been expanded into pharmacogenomic studies that examine individual genetic differences (in both tumor and uninvolved tissue) affecting therapeutic response and host toxicity (Diasio & Johnson, 2000; Ishikawa et al., 1999; Kirihara, Yamamoto, Toge, & Nishiyama, 1999; Lenz et al., 1998; Salonga et al., 2000). However, accurate determination of gene expression levels requires a ubiquitously expressed internal standard or housekeeping gene (HKG). Several techniques that are currently used to evaluate gene expression levels (including RNA protection assays, Northern blot analysis, and quantitative real-time polymerase chain reaction [Q-RT-PCR]) use HKGs to normalize the amount of total RNA in unknown samples. HKG variability in expression may lead to errors when interpreting experiments involving quantitation of gene expression, particularly in pharmacogenomic studies where minimal differences in expression may be important. Several recent studies have suggested that two of the most widely used HKGs, GAPDH and β-actin, demonstrate variability between different tissues and/or diseased states (Harrison et al., 2000; Suzuki, Higgins, & Crawford, 2000; Thellin et al., 1999; Yuan, Rosenberg, Ilieva, Agapitos, & Duguid, 1999; Zhong & Simons, 1999).

In the present study, total RNA was extracted from synchronous tissue samples of 10 patients. This included primary colorectal carcinoma, liver metastases, and corresponding normal tissue samples. Q-RT-PCR analysis was then used to evaluate the variability in gene expression among 15 HKGs (see Table 1) in order to determine the extent of variability in gene expression between normal and carcinomatous tissue. The dramatic differences in HKG expression between colon and liver tissues observed in this study emphasize the importance of selecting the proper HKG when normalizing RNA concen-

Table 1

Housekeeping Genes (HKGs) Examined

HKG	Function
GAPDH	glycolitic enzyme; (widely cited and commonly used as an HKG).
β-Actin	cytoskeletal protein involved in cell locomotion; (also widely used as an HKG).
S9 ribosomal	Ribosomal subunit; (nonmetabolic protein and expressed messenger RNA which does not require multiple dilutions before quantitative analysis like 18S rRNA does).
Ubiquitin	Involved in eukaryotic proteolysis, expressed in most eukaryotic cells and suggested to have minimal variation in expression according to recent unpublished microarray studies.
β-Tubulin	Member of the tubulin family of structural proteins; (a nonmetabolic protein previously used as an internal standard in several studies).
18S ribosomal RNA	Makes up 80% of total RNA, transcribed by a different polymerase from messenger RNAs, and its level is less likely to fluctuate with the test sample. It is quite an abundant target and requires multiple dilutions in order to perform quantitative analysis.
GUS	β-Glucuronidase is a relatively abundant glycoprotein expessed constitutively in many tissues, acting as an exoglycosidase in lysomes.
HPRT	Hypoxanthine ribosyl transferase is located on the X chromosome and is constitutively expressed at low levels, playing an important role in the metabolic salvage of purines in mammals.
β-2m	β-2 Microglobulin is involved with immune response. It is moderately abundant and expressed in most tissue types.
TBP	TATA-binding protein is constitutively expressed in many tissues and in cells at low levels; it is required for transcription directed by RNA polymerases I, II, and III.
CYC	Cyclophilin is a major cellular component found in all cells of wide phylogenetic distribution, originally isolated as the main cyclosporine A-binding protein.
huPO	Human acidic ribosomal protein is a moderately abundant protein found in most tissue types.
PGK	Phosphoglycerokinase is a key enzyme involved in glycolysis following GAPDH.
hTfR	Human transferrin receptor mediates cellular iron uptake and is expressed at low levels in both tissues and cells.
G6PDH	glucose-6-phosphate dehydrogenase; an additional metabolic enzyme and rate-controlling enzyme of the hexose monophosphate shunt.

Note. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Cyc = cyclophilin; PGK = 3-phosphoglycerokinase; HPRT = hypoxanthine-guanine phosphoribosyl transferase; TBP = TATA-binding protein; hTfR = human transferring receptor; GUS = β-glucuronidase; huPO = Human acidic ribosomal protein.

trations in Q-RT-PCR analyses. The approach described in this study to examine HKG variability can be applied to other tissue types and/or diseased states and may facilitate future analysis of gene expression quantitation.

Materials and Methods

Tissue Preparation

Following an institutionally approved protocol, informed consent was obtained from Stage 4, colorectal cancer patients undergoing surgical resection. Synchronous, colorectal carcinomatous, liver metastasis, and corresponding normal tissue samples were acquired. Prior to RNA extraction, all tissue obtained was reviewed by a surgical pathologist, snap frozen, and stored at -70 °C.

RNA Extraction

Total RNA was purified from tissue samples as previously described (Johnson, Wang, Smith, Heslin, & Diasio, 1999). The concentration was calculated spectrophotometrically by absorbance at 260 nm measurement and adjusted to 500 ng/μl. RNA integrity (degradation) was verified by electrophoresis and ethidium bromide staining on a 1% agarose gel.

Densitometric Quantitation of RNA

A standard curve was generated using known concentrations (750, 500, 250, 100, and 50 ng/μl) of authentic (human colon and liver) total RNA (purchased from Clontech, Palo Alto, CA). The concentration of each unknown patient sample (originally determined as 500 ng/μl by absorbance of 260-nm measurement) was confirmed by linear

regression analysis from the standard curve using densitometry of the 18S RNA band. Prior to Q-RT- PCR analysis, patient total RNA samples were diluted 1:100 with RNase free water (to a final concentration of 5 ng/μl). Normalizing the concentration of all tissue samples to 5 ng of total RNA was performed to ensure that differences in expression in the various tissues were due to HKG expression and not to differences in RNA concentrations.

Primers and Probe Design and Optimization

The primers and probes for GAPDH (GenBank Accession No. NM_002046) and β-actin (NM_001101) were purchased from Applied Biosystems and utilized according to manufacturer's instruction (Applied Biosystems, Foster City, CA). Primers and probes for 18S ribosomal RNA, GUS (NM_000181), HPRT (M31642), β-2m (V00567), TBP (M34960), CYC (Y00052), huPO (NM_001002), PGK (NM_000291), and huTfR (M11507) were included in the ABI TaqMan human endogenous control plates. These plates contain preoptimized primers and probes and were used as directed by the manufacturer (Applied Biosystems, Foster City, CA). The primers and probe for G6PDH (XM_013149) were obtained from a ready-to-use reaction primer-and-hybridization probe mixture provided by Roche Molecular Biochemicals (Mannheim, Germany) and used according to manufacturer's instruction.

The primers and probes for S9 (NM_001013), ubiquitin (AB009010), and β-tubulin (AF141349) were designed in our laboratory using the Primer Express software (Applied Biosystems, Foster City, CA). All primers and probes with the exception of G6PDH were synthesized and high performance liquid chromatography-purified by Applied Biosystems (Foster City, CA). The primer concentrations were optimized for S9,

ubiquitin, and β-tubulin by varying concentrations of both forward and reverse primers (50, 100, 150, 200, 250, and 300 nM) in order to determine the minimum primer concentration which would generate the maximum ΔR_n (difference between baseline and maximal fluorescence of sample). Optimum probe concentration was determined by running the reactions under the optimum primer concentrations and varying the probe concentrations (50, 100, 150, 200, 250, 300, 350 nM). The probe concentration that generated the lowest C_t (threshold cycle defined as the fractional cycle number at which the amount of the amplified target reaches a fixed threshold) was used as the optimum probe concentration (Johnson et al., 1999; User Bulletin No. 2, 1997).

Quantitative Polymerase Chain Reaction Analysis

Using an ABI PRISM 7700 sequence detection system, the log-linear phase of amplification was monitored to obtain C_t values for each RNA sample. The comparative C_t method was employed to determine the variation between normal and carcinomatous tissues among the 15 HKGs relative to a 5-ng, normal liver tissue standard designated as the calibrator (Johnson et al., 1999). Briefly, by subtracting the average C_t values of the calibrator from the average C_t values obtained from the patient samples, quantitative data relative to the calibrator were obtained (Johnson et al.; User Bulletin No. 2, 1997).

Patient samples were amplified with each specific HKG primer and probe and the HKG variability of the patient samples was derived from the difference between the average calibrator C_t values and the tissue samples (ΔC_t = average C_t of calibrator–average C_t of unknown samples; User Bulletin No. 2, 1997). Since each patient RNA sample (based on spectrophotometric and densitometric analysis) contains 5 ng of total RNA, the variation between carcinomatous tissues relative to their corresponding normal tissues re-

flects the variation among HKG expression. All reactions were run in triplicate along with an additional reaction in which reverse transcriptase was omitted to allow for assessment of genomic DNA contamination in each RNA sample.

Statistical Methods

To evaluate differences in expression of the HKGs in normal and malignant tissues, two-tailed *t* tests were performed where α was set at a value of 0.05.

Results

Densitometric Analysis and Quantitation of Total RNA

Each of the patient RNA samples was examined for degradation and equal 18S ribosomal RNA band intensity by electrophoresis and densitometric analysis, respectively, as shown in Figure 1A. Linear regression analysis from the standard curve confirmed that each patient sample contained 500 ng/µl of total RNA (as originally determined by absorbance of 260 measurement; Figure 1B). All spectrophotometric and densitometric concentration determinations varied less than 6%. Similar analyses were performed with the 10 normal and colorectal carcinomatous tissue samples (data not shown).

Optimization of Primers and Probes

Initial studies focused on optimizing primer and probe concentrations for S9, ubiquitin, and β-tubulin. The remaining primer and probe concentrations were used following manufacturer's instructions (see Materials and Methods).

The final optimized buffer composition for S9, ubiquitin, and β-tubulin was as follows: in a 25-µl reaction volume 2.5 µl of 10X TaqMan Buffer, 3.5 mM $MgCl_2$, 300

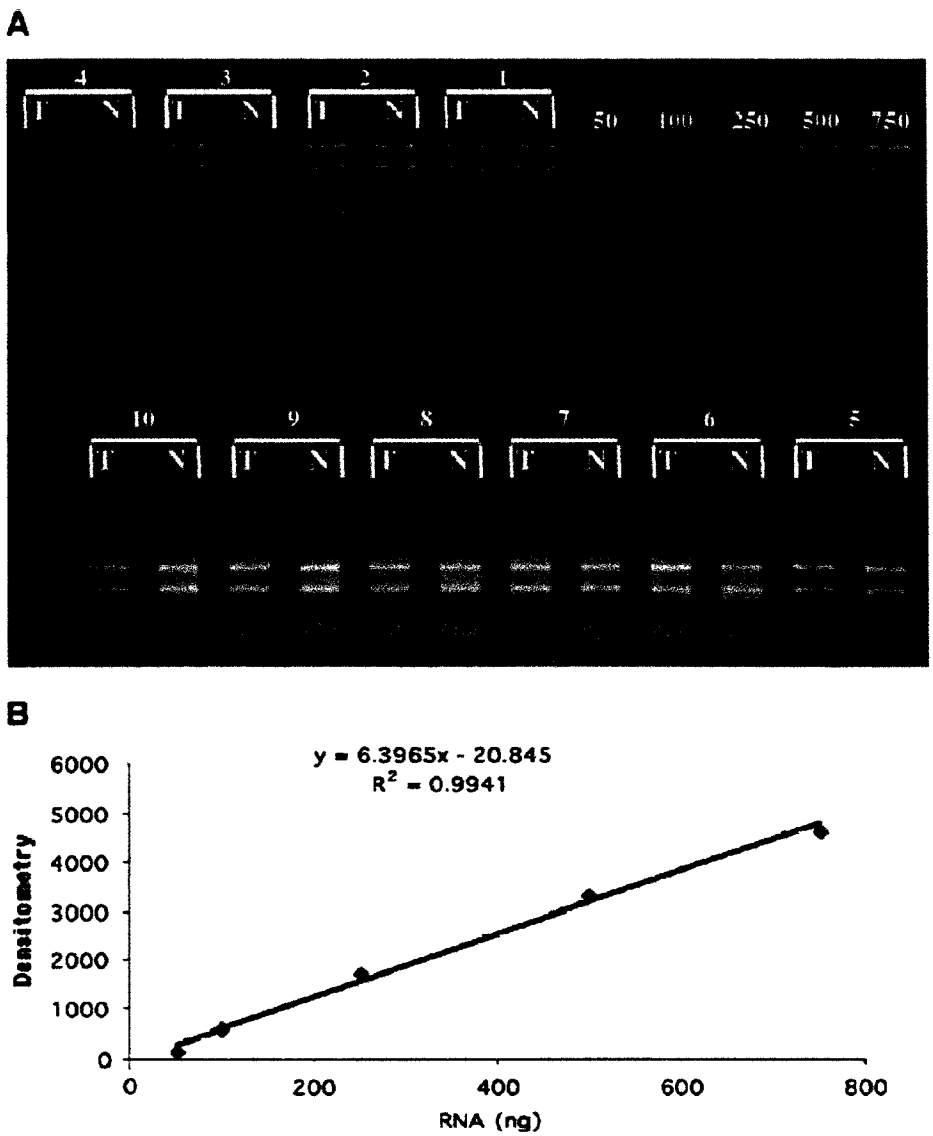


Figure 1. A representative gel containing authentic total RNA standards and 10 RNA patient samples and the standard curve produced by densitometric analysis of the 18S ribosomal RNA band from the authentic RNA standards. (A) A representative gel containing authentic total RNA standards (750, 500, 250, 100, and 50 ng) and 10 RNA patient samples (designated 1-10 where *T* is carcinoma and *N* is normal) is shown. Normal and corresponding liver metastasis from the same patient demonstrate similar 18S RNA band intensity (indicating equivalent amounts of total RNA) across all samples. Minimal degradation was observed. (B) The standard curve produced by densitometric analysis of the 18S ribosomal RNA band from the authentic RNA standards is shown. Linear regression analysis ($Y = 6.397x - 20.845$, R^2 value = 0.99) was performed to confirm that each unknown patient sample contained 500 ng/μl of total RNA (as originally determined by absorbance of 260-nm measurement). Because the concentration of all tissue samples was normalized to 5 ng of total RNA, differences in expression in the various tissues do not reflect differences in RNA amounts, but rather house keeping gene expression.

μM deoxyribonucleotides (2'-Deoxyadenosine 5'-triphosphate, 2'-Deoxycytidine 5'-triphosphate, 2'-Deoxyguanosine 5'-triphosphate, 2'-Deoxyuridine 5'-triphosphate), 0.8% glycerol, and the final optimized primer and probe concentrations for each HKG. The cycling conditions were performed as previously described (Johnson et al., 1999). Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template-control reactions were performed (data not shown).

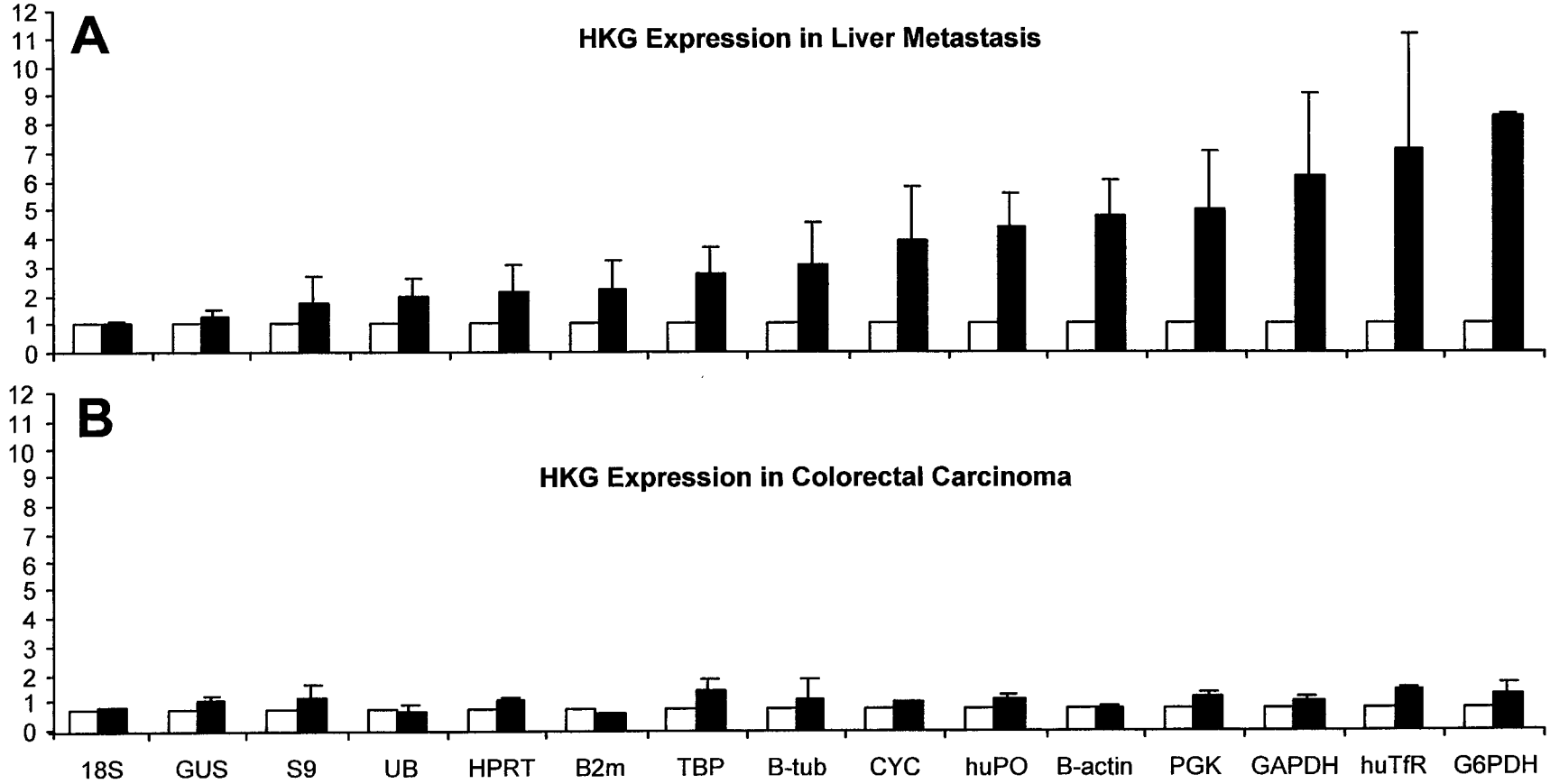
Quantitation of HKG Expression in Human Tissues Using Q-RT-PCR

The average values of the expression levels for each HKG and the standard deviations are shown in liver (Figure 2A) and colorectal tissues (Figure 2B). As summarized in Figure 2A, among the 15 HKGs, G6PDH, hTfR, GAPDH, and PGK showed the highest variability in expression in the liver metastatic tissues (compared to normal liver tissue), demonstrating 8.3-, 7.2-, 6.2-, and 5-fold expression increases, respectively. In contrast, 18S, GUS, and S9 expression remained relatively constant (less than 2 fold in liver metastasis). Ubiquitin, HPRT, β-2m, TBP, β-tubulin, CYC, huPO, and β-actin expression demonstrated intermediate variability in expression (ranging from 2 fold to 4.8 fold) between liver metastasis and normal liver tissue expression (Figure 2A). In normal and colorectal carcinomatous tissue, the 15 HKGs evaluated demonstrated variations of less than 2 fold (Figure 2B).

Discussion

The recent introduction of Q-RT-PCR represents a significant advancement in our ability to accurately quantitate gene expression levels in small (needle biopsy-sized)

Figure 2. Comparative expression as determined by quantitative real-time polymerase chain reaction for each housekeeping gene (HKG) is shown. (A) Normal (uninvolved; white bars) and metastatic (black bars) liver tissues. (B) Normal (uninvolved; white bars) and colorectal (black bars) carcinomatous tissues. The standard deviations for each housekeeping gene were calculated based on quantitative real-time polymerase chain reaction analysis of tissue samples for each of the normal and carcinomatous tissues ($n = 10$). Fold increases above 1 indicate housekeeping gene overexpression. These data were also analyzed separately with matched (normal vs. carcinomatous), individual tissue samples, and similar results were obtained. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Cyc = cyclophilin; PGK = 3-phosphoglycerokinase; HPRT = hypoxanthine-guanine phosphoribosyl transferase; TBP = TATA-binding protein; hTfR = human transferring receptor; GUS = β-glucuronidase; huPO = human acidic ribosomal protein.



tissue samples. One critical requirement in the accurate quantitation of gene expression is the availability of a ubiquitously expressed HKG to normalize the amount of total RNA in each sample. The purpose of this study was to use Q-RT-PCR to evaluate the variation in expression among 15 different HKGs in normal versus carcinomatous tissues and determine the optimal HKG for pharmacogenomic studies of primary colon cancer as well as colon cancer metastatic to the liver.

Comparative analysis of 15 HKGs (Table 1) in normal versus carcinomatous tissues revealed dramatic differences between liver and colon tissues (Figure 2). Primary carcinomatous colon samples demonstrated less than 2-fold differences in expression of each of the 15 HKGs compared to normal colon tissue. In contrast, there was significant variation in the expression of the 15 HKGs in carcinomatous and normal liver samples. These data emphasize the need to evaluate the variability of each HKG in different tissue types.

As a group, HKGs that code for metabolic enzymes (PGK, GAPDH, and G6PDH) demonstrated the highest overexpression in carcinomatous versus normal liver samples (see Figure 2A). The nonmetabolic (ubiquitin, GUS, β-2m, TBP, CYC, huTfR) and structural (β-tubulin, β-actin) HKGs demonstrated less variation than the metabolic HKGs. Ribosomal HKGs such as 18S and S9 demonstrated the least variability (less than 2 fold) in liver metastases compared to normal liver. This may result from their lack of involvement in cellular metabolism (Finnegan, Goepel, Hancock, & Goyns, 1993; Kotter & Entian, 1995). GUS, involved in carbohydrate metabolism, also demonstrated minimal variability of expression (1.3 fold) between liver metastasis and normal liver. These data demonstrate that the function of an enzyme cannot be used as an indicator of HKG vari-

ability, thus further emphasizing the need to validate a particular HKG in the tissue of interest prior to analysis.

In summary, these data show that 18S, S9, and GUS are the least variable of the 15 HKGs examined in normal liver and carcinoma metastatic to the liver and are also appropriate HKGs for normal and carcinomatous, colorectal tissues. Having a validated HKG should facilitate comparisons between normal and carcinomatous tissue by diminishing potential errors introduced by variable HKG expression. The approach described in this study could be used to validate other HKGs in any tissue type and/or disease state.

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INDUCTION OF THYMIDINE PHOSPHORYLASE IN BOTH IRRADIATED AND SHIELDED, CONTRALATERAL HUMAN U87MG GLIOMA XENOGRAFTS: IMPLICATIONS FOR A DUAL MODALITY TREATMENT USING CAPE AND IRRADIATION

by

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Abstract

In the United States, tumors of the central nervous system remain the 3rd leading cancer-related cause of death in young adults with a median survival time of <1 year. A recent case study suggested that capecitabine (a novel, fluoropyrimidine prodrug) may be effective in the treatment of brain metastases. Pharmacogenomic studies have correlated the antitumor response to capecitabine with the expression of the drug metabolizing enzymes thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase. In the current study, we examined TP and dihydropyrimidine dehydrogenase expression in normal human brain tissues and in glioblastoma multiforme, the most common and malignant type of brain tumor. Because previous reports suggest a tumor necrosis factor-α-mediated increase in TP expression after irradiation (a current standard of care for glioblastoma multiforme), we also examined the effect of irradiation on the expression of TP, dihydropyrimidine dehydrogenase, and tumor necrosis factor-α in both irradiated and lead-shielded contralateral U87MG glioma xenografts within the same animal. Expression levels were determined using real-time quantitative polymerase chain reaction as described previously. Results demonstrate an ~70-fold increase in TP messenger RNA (mRNA) levels 4 days after irradiation, relative to initial control levels. Interestingly, TP RNA in the lead-shielded tumors (contralateral to irradiated tumors) increased ~60 fold by Day 10 relative to initial control levels. Elevated TP levels were sustained for 20 days in irradiated xenografts but began to decrease after 15 days in the shielded/contralateral tumors, returning to baseline by 20 days. TP mRNA levels in normal mouse liver were unaltered, suggesting a tumor-associated effect. Tumor necrosis factor-α mRNA levels did not increase after irradiation; therefore, mRNA expression of 11 additional cytokines (interleukin [IL]-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, and inter-

feron-γ) in both the irradiated and shielded xenografts was quantitated. Results demonstrated increased levels of interferon-γ, IL-10, and IL-1α by 6.3, 3.7, and 1.6 fold, respectively, in irradiated tumors only. Dihydropyrimidine dehydrogenase mRNA levels did not change after irradiation. The tumor-associated induction of TP in irradiated and lead-shielded tumors within the same animal may have significant implications for the combined modality treatment of cancer patients with capecitabine in conjunction with radiotherapy and may apply to the treatment of distant tumors and or metastatic disease.

Introduction

In the United States, tumors of the central nervous system remain the most prevalent solid neoplasm of childhood and the third leading cancer-related cause of death in adolescents and adults between the ages of 15 and 34 years (Galanis & Buckner, 2000). Unfortunately, despite over 50 years of research (including highly aggressive therapeutic approaches), the median survival of patients with malignant brain tumors remains <1 year (Macarthur & Buxton, 2001; Prados & Levin, 2000). Glioblastoma multiforme (GBM) remains the most common and malignant type of brain tumor and is characterized by an unusual resistance to treatment with both radiation and chemotherapy. Nitrosourea agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea are frequently used concurrently with radiotherapy (Brem et al., 1995; Hildebrand, Sahmoud, Mignolet, Brucher, & Afra, 1994); unfortunately, severe dose-related toxicity limits the usefulness of these compounds (Subach et al., 1999). Although chemotherapy agents have not generally demonstrated survival benefit in this disease, a recently published case report suggested that capecitabine (CAPE) might be an effective treatment for brain metastases (Wang, Yung, Royce, Schomer, & Theriault, 2001).

CAPE is a recently introduced oral prodrug that is converted into 5-fluorouracil (5-FU) by three sequential enzymatic steps. Thymidine phosphorylase (TP) is the final and rate-limiting enzyme responsible for CAPE activation (Bajetta, Carnaghi, Somma, & Stampino, 1996; Budman et al., 1998; Ishikawa, Fukase, Yamamoto, Sekiguchi, & Ishitsuka, 1998). Once converted into 5-FU, metabolism results in either: (a) anabolism into cytotoxic nucleotides, which are ultimately responsible for tumor cell death; or (b) catabolism into biologically inactive metabolites that are excreted in the urine and bile (Heggie, Sommadossi, Cross, Huster, & Diasio, 1987). Pharmacokinetic studies have shown that the amount of 5-FU available for anabolism is determined by the extent of its catabolism (Diasio, Beavers, & Carpenter, 1988; Heggie et al.). Thus, a delicate balance exists between the enzymatic activation of CAPE into 5-FU (catalyzed by TP) and its catabolic elimination (catalyzed by dihydropyrimidine dehydrogenase [DPD]). Several studies evaluating CAPE have demonstrated that intratumoral levels of TP and DPD (expressed as a TP/DPD ratio) are the best indicators of tumor response with increased efficacy characterized by high TP expression (increased conversion of CAPE into 5-FU) and low DPD expression (decreased inactivation of 5-FU; Ishikawa, Sekiguchi, Fukase, Sewada, & Ishitsuka, 1998; Schuller et al., 2000; Tsukamoto et al., 2001). In addition, a recent clinical study suggests that high TP expression is a good indicator of disease-free survival for those patients taking 5-FU prodrug-based chemotherapy, with patients expressing high TP and low DPD demonstrating the best disease-free survival (Nishimura et al., 2002).

Induction of TP has been suggested as a potential method of increasing efficacy of CAPE. Several cytokines (interleukin [IL]-1-α, tumor necrosis factor [TNF]-α, interferon [IFN]-γ) and anticancer drugs (paclitaxel, docetaxel, cyclophosphamide) have been

shown to increase TP expression (Eda et al., 1993; Endo et al., 1999; Sawada et al., 1998). Of particular importance, irradiation has been shown to result in increased TP expression with a concurrent increase in CAPE efficacy (Sawada, Ishikawa, Sekiguchi, Tanaka, & Ishitsuka, 1999). Because irradiation remains a current standard of care for the treatment of GBM, the addition of CAPE represents a rational and potentially synergistic combination.

In this study, we quantitated TP and DPD messenger RNA (mRNA) expression levels in GBM and normal human brain tissue. The effect of irradiation on the expression of TP, DPD, and several cytokines in contralateral irradiated and lead-shielded U87MG glioma xenograft tumors was also examined. The combined modality treatment of CAPE and irradiation may have significant implications for tumor response, which could extend to distant or metastatic tumors and, ultimately, result in prolonged patient survival.

Materials and Methods

Tissue Preparation

After an Institutional Review Board-approved protocol (X980409003), GBM and normal brain tissue samples were obtained from cancer patients undergoing surgical resection. Tissues used for RNA extraction had been snap frozen and stored at -70 °C. A representative sample of the tissue obtained was fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin so that it could be examined by a neuropathologist to establish a diagnosis.

RNA Extraction

Total RNA was isolated as described previously (Blanquicett, Johnson, Heslin, & Diasio, 2002; Johnson, Wang, Smith, Heslin, & Diasio, 2000). All sample concentrations were calculated spectrophotometrically at absorbance at 260 nm and diluted to a final concentration of 20 ng/μl in RNase-free water containing 12.5 ng/μl of total yeast RNA (Ambion, Austin, TX) as a carrier.

Real-Time Quantitative Polymerase Chain Reaction

Expression levels were determined using an ABI 7700 Sequence Detection System as previously described by our laboratory (Blanquicett et al., 2002; Johnson et al., 2000). Before sample analyses, housekeeping gene variation was determined for GBM and normal brain tissue as previously described by our laboratory (Blanquicett et al.). The primers and probes for human TP, DPD, TNF-α, S9 ribosomal RNA (GenBank Accession No. NM001953, U20938, M10988 and NM001013, respectively) and mouse TP (GenBank accession no. AW744006) were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The sequence and optimum primer/probe concentrations are shown in Table 1. Expression levels were calculated using the relative standard curve method as described previously (Blanquicett et al.; Johnson et al., 2000). All reactions were run in triplicate, and standard curves with correlation coefficients falling <0.98 were repeated. Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template control reactions were performed.

Table 1

Primer and Probe Sequences and Optimal Concentrations

Target	Sequence	Optimal conc. (nM)
TP (human)		
Fwd	TCCTGCGGACGGAATCC	100
Probe	**FAM**-CAGCCAGACATGTGACAGCCA-CCGT-**TAMRA**	200
Rev	TGAGAATGGAGGCTGTGATGAG	200
TP (mouse)		
Fwd	CGGCCAGAGTGCAAAGCT	200
Probe	**FAM**- CAGCATACAGGATCCCATCAG-CAGGAA-**TAMRA**	200
Rev	TCCACAGTGGCTGTCACATCTC	300
TNF-α		
Fwd	GGAGAAGGGTGACCGACTCA	300
Probe	**FAM**-CGCTGAGATCAATCGGCCCGACTAT-**TAMRA**	250
Rev	TGCCCAGACTCGGCAAAG	200
S9		
Fwd	ATCCGCCAGCGCCATA	100
Probe	**FAM**-AGCAGGTGGTGGTGAACATCCCGT CCTT-**TAMRA**	300
Rev	TCAATGTGCTTCTGGGAATCC	100
DPD		
Fwd	CCAAAGGCAGTAAAGCAGGAA	300
Probe	**FAM**-TGCGCCTGTCACTCTCCATTGCC-**TAMRA**	25
Rev	TCACGACTCCCCGTATCGA	100

Note. Conc. = concentration; TP = thymidine phosphorylase; TNF = Tumor necrosis factor; S9 = ribosomal subunit; DPD = dihydropyrimidine dehydrogenase.

Cell Culture

U87MG glioma cells (purchased from the American Type Culture Collection, Manassas, VA) were maintained in stationary monolayer cultures at 37 °C and 5% CO_2 in a humidified atmosphere using a 50:50 mixture of Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F12 supplemented with 7% heat-inactivated fetal bovine serum and 2.6 mM L-glutamine. All cell cultures were maintained in antibiotic-free condi-

tions and regularly checked for *Mycoplasma* contamination using a polymerase chain reaction-based kit. Near confluent (75%) monolayers of cells were harvested by brief exposure to 0.05% trypsin/0.53 mM EDTA (Life Technologies, Inc. Gaithersburg, MD). Harvested cells were pelleted (200 x *g*, 8 min at ambient temperature) in complete medium and resuspended in serum-free medium. Viable cells were counted using a Neubauer hemacytometer using trypan blue (0.4%) exclusion. To determine whether IFN-γ induced TP transcription, 0.5 ng/μl IFN-γ were added to 1 x 10^6 U87MG glioma cells and omitted in the control population. Cells were incubated for 24 hr and collected as described above for RNA isolation.

Human Cancer Xenograft Preparation and Irradiation

Athymic, nude NCr mice (nu/nu) were anesthetized with ketamine/xylozine and subcutaneously. injected bilaterally into hind flanks with a suspension of 5 x 10^6 U87MG glioma cells. Tumors were allowed to develop between 200 and 400 mm^3 in size. Mice were randomized into control and treatment groups, and one of the glioma tumor-bearing flanks of the treated group was irradiated, whereas the rest of the body (including the tumor in the opposite flank) was lead shielded (Figure 1). Irradiation was carried out using a ^{60}Co teletherapy X-ray unit (Picker, Cleveland, OH) at a dose of 8.5 Gy. Mice in control groups were anesthetized before the irradiation but were not irradiated. Mice were sacrificed between 0-20 days after irradiation.

Cytokine Expression

The effect of irradiation on the expression of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN- γ, and TNF-α) in the U87MG

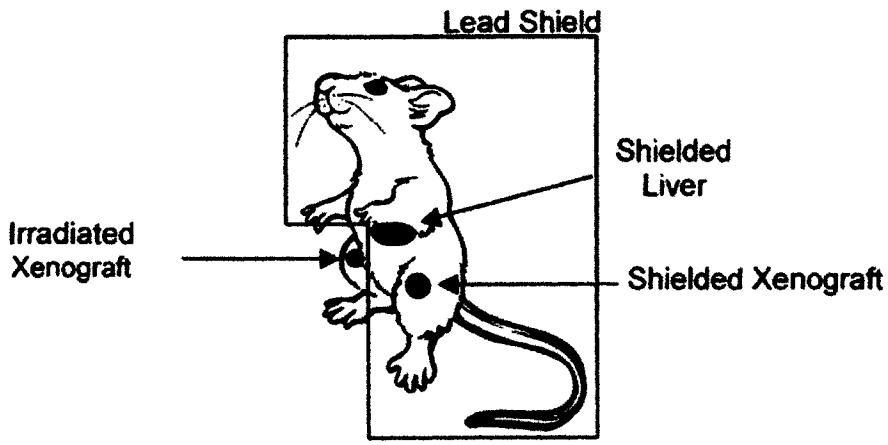

Figure 1. Schematic of mouse xenograft location(s) and irradiation. One of the two flanks containing a U87MG glioma xenograft was irradiated, whereas the contralateral flank (containing the other U87MG xenograft) as well as the body of the mouse (including the mouse liver) was lead shielded.

glioma xenografts was examined using a Taqman Cytokine Gene Expression Plate I (Applied Biosystems) according to manufacturer's instruction. Nonirradiated U87MG glioma xenografts in control mice were used as the calibrator.

Statistical Analyses

Statistical analyses were performed using Student's *t* test where differences were considered to be significant when $p < 0.05$.

Results

Quantitation of TP and DPD Expression in GBM and Normal Brain Tissues

Before quantitation using real-time quantitative polymerase chain reaction, we examined the variability of 12 housekeeping genes in GBM and normal brain tissues as previously described by our laboratory (Blanquicett et al., 2002). The housekeeping genes examined include the following: *18S ribosomal RNA*; *ß-glucuronidase*; *ß2-microglobulin*; *ß-actin*; *S9 ribosomal RNA*; *acidic ribosomal protein*; *TATA binding protein*; *transferrin receptor*; *glyceraldehyde 3-phosphate dehydrogenase*; *cyclophilin*; *phosphoglycerokinase*; and *hypoxanthine ribosyl transferase*. On the basis of these analyses, the ribosomal S9 gene (which demonstrated <1.5-fold variation in expression between normal and tumor tissues) was used to normalize the amount of total RNA in each sample (data not shown).

As shown in Figure 2, TP expression is higher in GBM (T) as compared with normal brain tissue (N) with a mean difference of 6.2 ($SD = 7.2$; $p = 0.13$). Furthermore, this data suggests more variability of TP expression among tumor samples (ranging from

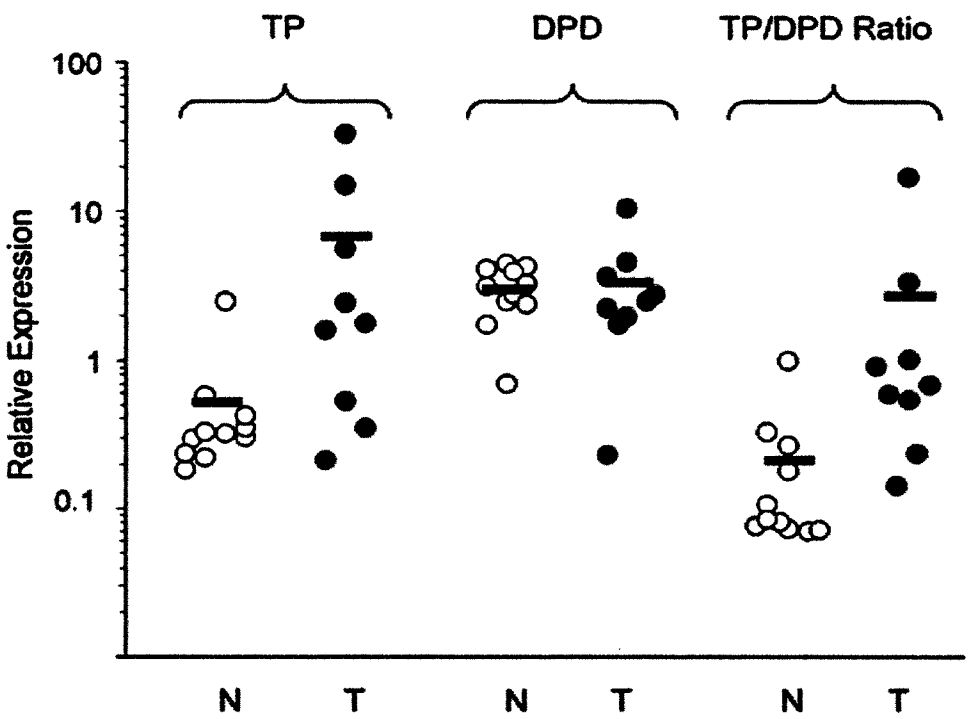

Figure 2. Expression of thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) in glioblastoma multiforme (GBM) (T) and normal brain (N) tissue. As shown above, TP expression is higher in GBM (n = 9) as compared with normal brain (n = 11) with a mean difference of 6.2 (SD = 7.2; p = 0.13). There was no significant difference in DPD expression levels in the same tissue samples (mean difference of 0.32; SD = 2.13; p = 0.76). The average TP/DPD ratio demonstrated in GBM (2.69) is ~16-fold higher than that of normal brain tissue (0.17) and is primarily because of TP overexpression in GBM. This profile should result in selective conversion of CAPE in tumor compared with normal brain tissues (16-fold higher TP/DPD ratio in GBM relative to normal brain). Error bars have been incorporated into this figure but are so small as to be obscured by the data point.

a low of 0.21 to a high of 33.01) as compared with normal brain tissue (ranging from a low of 0.18 to a high of 2.47). There was no significant difference in DPD levels between normal and tumor tissue samples (mean difference of 0.32; $SD = 2.13$; $p = 0.76$). The average TP/DPD ratio demonstrated in GBM (2.69) is ~16-fold higher than that of normal brain tissue (0.17). The higher TP/DPD ratio in GBM is primarily because of higher expression of TP in GBM (6.72) compared with normal brain tissue (0.52) because there was no significant difference in DPD levels in the same tissues (3.33 and 3.01, respectively).

TP, DPD, and TNF-α Expression in U87MG Xenografts

As described in the Materials and Methods and illustrated in Figure 1, athymic NCr nude mice were given injections of glioma cells in both hind flanks and allowed to develop tumors. One of the tumor-bearing flanks of the treated group was irradiated, whereas the rest of the mouse was lead shielded (Figure 1). As shown in Figure 3A, TP mRNA levels did not increase during the first 24 hr after irradiation. However, by Day 2, TP mRNA levels in irradiated tumors rapidly increased, peaking at Day 4 with a ~70-fold increase relative to initial, nonirradiated tumors in control mice. As shown in Figure 3A, the increase in TP mRNA levels was maintained in irradiated tumors for up to 20 days after irradiation (mean difference of 39.31; $SD = 18.5$; $p < 0.001$) and was an average 10-fold higher than control (nonirradiated) tumors.

TP mRNA in the lead-shielded tumor (from the contralateral flank of the same mouse) did not increase during the first 8 days after irradiation (Figure 3B). However, by days 10 and 15, TP mRNA levels increased ~60 fold relative to initial TP mRNA levels in nonirradiated tumors in control mice (mean difference of 18.07; $SD = 16.2$; $p = 0.04$).

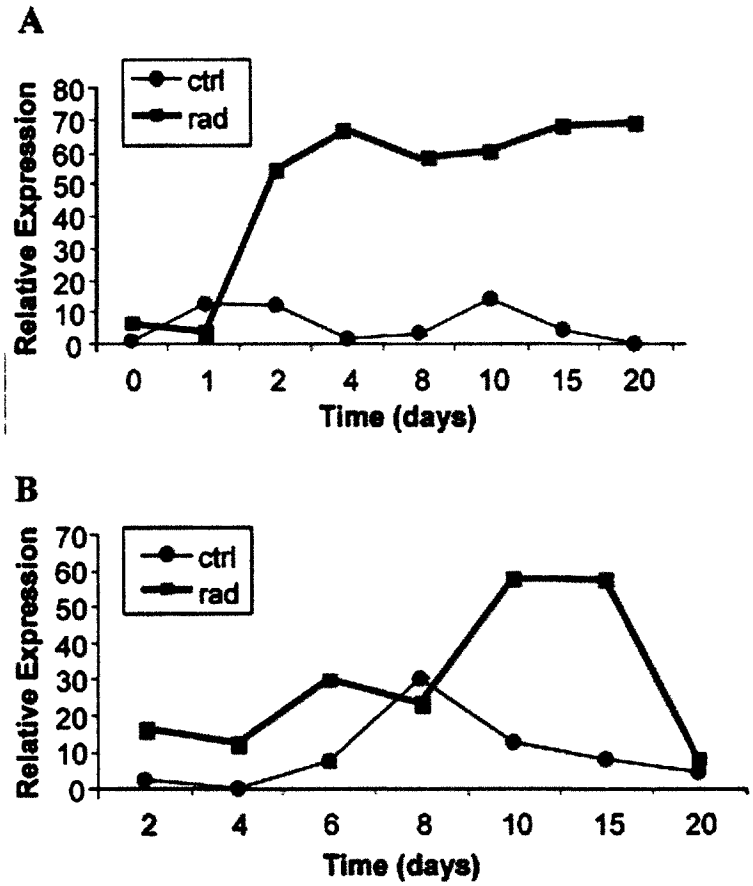

Figure 3. Thymidine phosphorylase expression in irradiated and shielded U87MG glioma xenografts in mice. (A) As shown above, thymidine phosphorylase messenger RNA levels increased ~70 fold in irradiated tumors (rad) relative to nonirradiated tumors in control (ctrl) mice, remaining elevated 20 days after irradiation. (B) Of particular interest, thymidine phosphorylase messenger RNA levels in the shielded tumors (in the same animal) increased ~60 fold (relative to initial control levels) 10 and 15 days after irradiation before dropping to control levels at 20 days. Error bars have been incorporated into this figure but are so small as to be obscured by the data point.

Unlike the irradiated tumors, TP mRNA levels in the shielded tumors decreased to control levels 20 days after irradiation (Figure 3B). No significant change in either DPD (mean difference of 0.13; $SD = 0.56$; $p = 0.61$) or TNF-α (mean difference of 0.45; $SD = 1.2$; $p = 0.41$) expression was observed in either the irradiated or shielded tumors relative to control tumors (data not shown).

TP Expression in Shielded Mouse Liver

To determine whether the increase in TP mRNA levels in the shielded xenografts was tumor associated, we examined mouse TP expression in liver tissue from treated (irradiated) and control (nonirradiated) mice. These analyses demonstrated no significant differences in mouse liver TP mRNA levels (mean difference of 0.21; $SD = 0.39$; $p = 0.24$) between irradiated mice and control mice (Figure 4) over the time course studied, suggesting that the increase in TP mRNA seen in the xenografts was tumor associated.

Cytokine Expression in U87MG Xenografts

The delay between irradiation and the increase in TP mRNA levels (4 and 10 days in irradiated and shielded xenografts, respectively) suggests a secondary-mediated induction of TP rather than a direct effect. Because several cytokines, which have been shown to increase after irradiation, have also been shown to increase TP, we evaluated the expression of 12 cytokines (including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN-γ, and TNF-α) at several days preceding elevated levels of TP mRNA (Days 0, 1, 2, and 4 in irradiated tumors and days 0, 4, 6, and 10 in shielded tumors). As shown in Figure 5A, irradiated xenografts demonstrated the greatest increase in expression of IFN-γ (6.3 fold) followed by IL-10 (3.7 fold) at 4 days after irradiation and

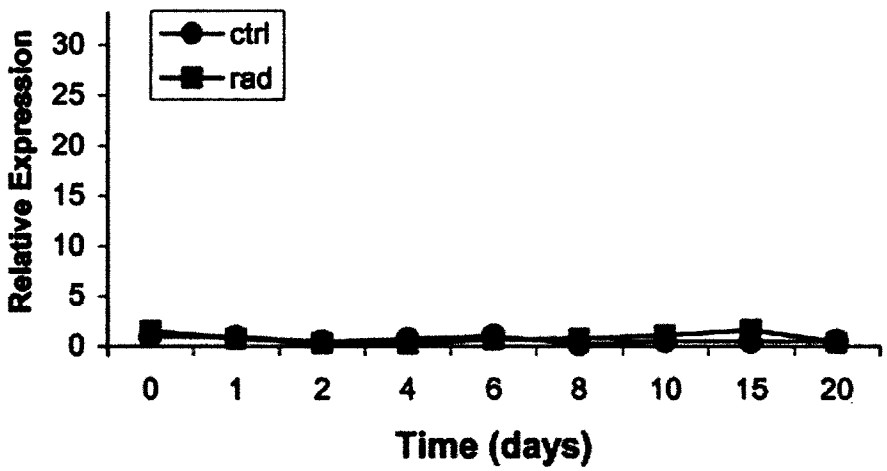

Figure 4. Thymidine phosphorylase expression in control (ctrl) and shielded (rad) normal mouse liver. Mouse thymidine phosphorylase messenger RNA expression in normal liver was evaluated in shielded and nonirradiated (ctrl) mice. As shown above, there is no significant difference between shielded and nonirradiated mouse liver thymidine phosphorylase messenger RNA levels. Error bars have been incorporated into this figure but are so small as to be obscured by the data point.

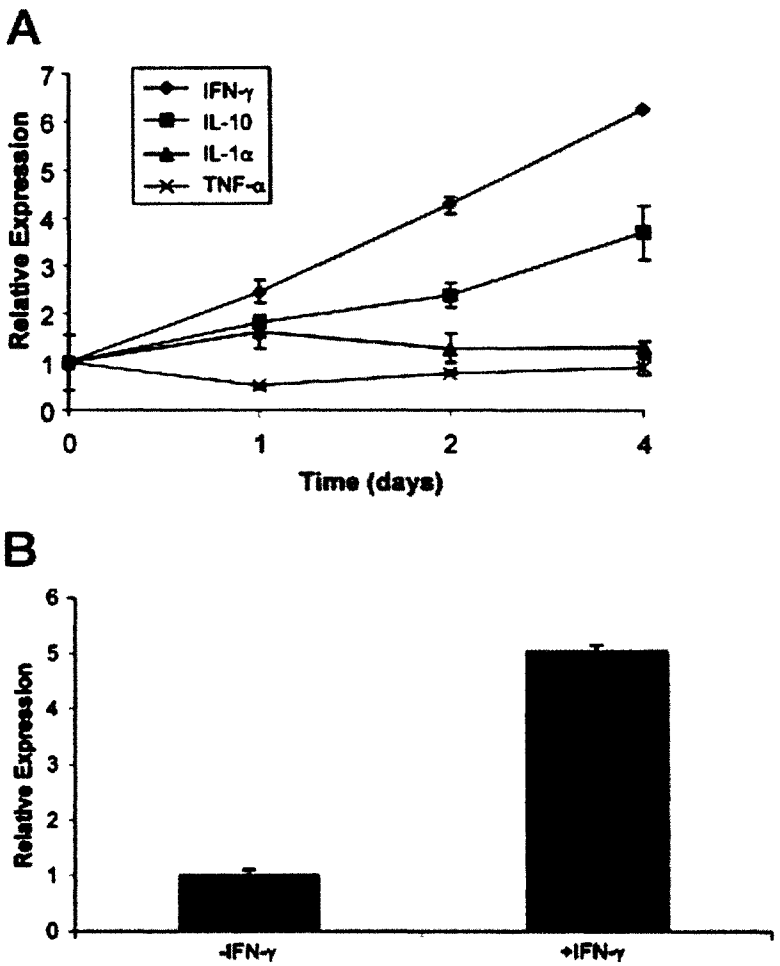

Figure 5. Effect of irradiation on messenger RNA expression of 12 cytokines and quantitation of thymidine phosphorylase in U87MG glioma cells without (-) and with (+) interferon (IFN)-γ. (A) Effect of irradiation on messenger RNA expression of 12 cytokines. Cytokine expression in irradiated tumors (Days 0-4 after irradiation) was determined for 12 cytokines using a TaqMan Cytokine Gene Expression Plate I as described in Materials and Methods. As shown above, IFN-γ demonstrated the greatest increase in expression (6.3 fold) followed by interleukin (IL)-10 (3.7 fold) in irradiated xenografts relative to control xenografts at 4 days after irradiation. IL-1α demonstrated a <2-fold increase in expression 24 hr after irradiation (1.6 fold), returning to baseline levels 2-4 days after irradiation. Tumor necrosis factor-α levels (included in the cytokine plate) did not increase above baseline. IL-2, IL-4, IL-5, and IL-8, which are not shown, did not demonstrate any increases in expression after irradiation. IL-1β, IL-12p35, IL-12p40, and IL-15 messenger RNA levels were beyond the limits of detection in all of the samples (data not shown). (B) Quantitation of thymidine phosphorylase in U87MG glioma cells without (-) and with (+) IFN-γ. TP expression (messenger RNA levels) increased 5 fold in the U87MG glioma cells treated with IFN-γ (+) as compared with untreated cells (-), suggesting a direct effect of IFN-γ on thymidine phosphorylase transcription.

relative to nonirradiated xenografts. IL-1α demonstrated a <2-fold increase in expression 24 hr after irradiation (1.6 fold), returning to baseline levels 2-4 days after irradiation. TNF-α levels (included in the commercially available cytokine plate) did not increase above baseline levels, agreeing with results from our independently designed primers and probes. Expression levels of IL-2, IL-4, IL-5, and IL-8 did not increase after irradiation but were detectable, whereas IL-1β, IL-12p35, IL-12p40, and IL-15 mRNA levels were undetectable in both irradiated and control xenografts.

Cytokine expression was also examined in contralateral, shielded tumors (see Figure 1) and compared with tumors in nonirradiated mice on Days 0, 4, 6, and 10. Of the 12 cytokines examined, TNF-α and IL-10 demonstrated <2-fold increases in expression on days 4 and 6. Although IL-8 and IL-1α mRNA levels were detected in the shielded tumors, no increases were observed with these cytokines (data not shown). The remaining cytokines were below the limits of detection (IL-1β, IL-2, IL-4, IL-5, IL-12p35, IL-12p40, IL-15, and IFN-γ).

Induction of TP Expression in Cultured U87MG Cells with IFN-γ

To determine whether IFN-γ alone can induce TP transcription, U87MG glioma cells were incubated with IFN-γ for 24 hr. As shown in Figure 5B, TP mRNA increased 5 fold compared to control cells.

Discussion

Despite highly aggressive therapeutic approaches, the median survival of 1 year for patients with malignant brain tumors has not appreciably changed in the last 50 years (Galanis & Buckner, 2000; Macarthur & Buxton, 2001; Prados & Levin, 2000). Recent

advances in the molecular analysis of tumor tissue that allow quantitation of drug-metabolizing enzymes have resulted in the ability to predict response to chemotherapy (Andratschke, Grosu, Molls, & Nieder, 2001; Diasio & Johnson, 2000; Johnson et al., 1999; Johnson et al., 2000; Mori et al., 2000; Salonga et al., 2000). Although previous studies have suggested that this pharmacogenomic approach can be used to increase efficacy through selection of a subpopulation of patients likely to respond to chemotherapy (Ishikawa et al., 1998; Mori et al.; Schuller et al., 2000; Tsukamoto et al., 2001), a broader approach would be to design novel therapies based on the molecular profile of the tumor type.

A recent study reported the effective use of CAPE for brain metastases originating from breast cancer (Wang et al., 2001). The partial response (shown by decreased lesion size and improved mental performance) was particularly noteworthy because previous hormonal treatment, whole brain irradiation, and systemic chemotherapy, including treatment with 5-FU, proved ineffective. In addition to providing a potentially effective treatment for brain metastases, this study demonstrated that: (a) tumor resistance to 5-FU does not preclude effective treatment with CAPE; and (b) CAPE can reach therapeutic concentrations in brain tumor tissues. Because response to CAPE has been correlated to intratumoral expression of TP and DPD (expressed as a TP:DPD ratio; Ishikawa et al., 1998; Mori et al., 2000; Schuller et al., 2000; Tsukamoto et al., 2001), we examined the expression of these drug-metabolizing enzymes in GBM and normal human brain tissues. In addition, the effects of irradiation on TP mRNA expression were examined in both irradiated and shielded, contralateral U87MG glioma xenografts.

As shown in Figure 2, the average TP/DPD ratio in GBM is ~16-fold higher than in normal brain tissue. This increased TP/DPD ratio is primarily because of higher TP ex-

pression in tumor compared with normal brain tissue. There was no significant difference in DPD expression levels between normal and tumor tissues. This distribution of TP and DPD should result in selective intratumor activation of CAPE (i.e., intratumoral 5-FU levels would be higher than normal tissue), whereas 5-FU clearance from tumor and normal tissues should be similar (equivalent DPD expression). Furthermore, these data suggest that in GBM and normal brain tissue samples, TP and DPD appear to be independent determinants of response with no apparent correlation in expression levels. Interestingly, the variability of TP expression is much higher in GBM as compared with normal brain tissue. However, whether this variability correlates to other factors such as tumor stage, location, or patient survival remains to be determined.

Recent studies have shown that it is possible to increase CAPE efficacy by induction of TP (Sawada et al., 1999). Although irradiation has been shown to result in increased TP levels with a concurrent increase in CAPE efficacy in colon, cervix, gastric, and breast cancer xenograft models (Sawada et al., 1999), the effects of irradiation in glioma xenograft models have not been examined before this study. As shown in Figure 3A, irradiation increased TP mRNA expression ~70 fold relative to initial control (nonirradiated) levels. Shielded contralateral tumors demonstrated a 60-fold increase in TP expression between 10 and 15 days after irradiation (Figure 3B). Because DPD levels were unaffected, this results in a 70- and 60-fold increase in the TP/DPD ratio for these tumors, respectively.

The current trend in treatment is to reduce the amount of neurotoxicity from whole brain radiation by focusing the irradiation to the area of residual tumor or the site of tumor excision (involved fields or intensity-modulated radiation therapy). However, recurrence is common, generally within a few centimeters of the original tumor site (just

beyond the irradiated field; Wild-Bode, Weller, Rimmer, Dichgans, & Wick, 2001). The contralateral xenograft model used in this study (Figure 1) was designed to represent invasive, metastatic, and/or micrometastatic tumors in humans. The induction of TP in shielded tumors that were not directly irradiated has potential implications for improving CAPE efficacy in patients with invasive tumors that were not directly irradiated during treatment. Furthermore, examination of shielded mouse liver TP (Figure 4) agrees with previous studies, suggesting that the induction of TP is a tumor-associated effect (Sawada et al., 1999). Interestingly, TNF-α mRNA levels did not increase after irradiation (Figure 5).

The molecular basis for induction of TP after irradiation remains to be elucidated. Although a previous study suggests that increased TNF-α levels after irradiation results in increased TP expression, no increase in TNF-α was observed in these glioma xenografts. Examination of Figure 3 reveals that elevated TP mRNA levels occurred at 4 and 10 days in irradiated and shielded tumors, respectively. This delayed increase in TP mRNA levels suggests a mediated and potentially complex mechanism with induction possibly involving a currently unidentified, soluble cell factor(s). The sustained increase in TP mRNA levels (particularly in irradiated tumors by up to 20 days) may also be suggestive of other mechanisms involved, such as stabilization of the TP mRNA transcript. Because IL-1α and IFN-γ have also been shown to induce TP (Eda et al., 1993; Endo et al., 1999; Sawada et al., 1998), we examined the expression of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN-γ, and TNF-α) in irradiated and shielded glioma xenografts (Figure 5A). Although significant changes were not detected in most of the cytokines examined, IFN-γ, IL-10, and IL-1α mRNA levels increased 6.3, 3.7, and 1.6 fold, respectively. In fact, IFN-γ has been shown to induce the highest levels

of TP expression relative to TNF-α and IL-1α in human macrophages with gamma-activated sequence elements in the TP promoter being essential for IFN-γ-dependent activation of the TP gene (Goto et al., 2001). In the current study, we demonstrate that IFN-γ induced TP mRNA levels in U87MG glioma cells (Figure 5B). Taken collectively, these studies suggest that the molecular basis for the induction of TP after irradiation may vary depending on tumor type with at least two mechanisms: (a) a TNF-α-independent mechanism (as demonstrated in the glioma xenograft models); or (b) a TNF-α-dependent mechanism (as demonstrated in the colon, cervix, gastric, and breast cancer xenograft models; Sawada et al., 1999). In addition, increased IFN-γ levels have been shown to have antitumor activity in recurrent gliomas by inhibition of angiogenesis, apoptosis of endothelial cells, suppression of glioma growth, and decreased cell proliferation (Dadie, Schartner, Vorpahl, & Preston, 2000; Knupfer, Poppenborg, Van Gool, Domula, & Wolff, 1997). These studies suggest the potential use of IFN-γ to induce TP expression in patients treated with CAPE (where irradiation is not a treatment option) or as an addition to the combination of CAPE and irradiation.

Previous studies have shown induction of IL-10 (a potent anti-inflammatory cytokine) after irradiation (Mizutani et al., 2002). In addition, immunohistochemical staining has suggested that elevated IL-10 protein levels correlate with elevated TP expression in a study examining oropharyngeal carcinoma (Fujieda, Sunaga, Tsuzuki, Fan, & Saito, 1999). The data presented in this study suggest that increased IL-10 expression precedes increased TP levels. Interestingly, IL-10 has been reported to suppress TNF-α (Denys et al., 2002). Additional studies will need to examine whether the increased levels of IL-10 observed in this study are related to the lack of increase in TNF-α mRNA levels in irradiated xenografts. The increase in IL-1α after irradiation that was observed in this study is

in agreement with studies that have also implicated IL-1α in TP up-regulation (Sawada et al., 1999). However, only a slight increase in IL-1α (<2 fold) during the first 24 hr after irradiation was observed. Future in vitro studies will examine the role of these cytokines in the molecular basis for TP induction after irradiation.

Although, chemotherapy has not yet emerged as a standard of care for brain tumors, a recent case study reports the successful treatment of brain metastasis with CAPE (Wang et al., 2001). Because ~24% of human cancers are known to metastasize to the brain (Pilkington, 1997), this may provide a new and potentially effective treatment option in a disease where incidence is increasing and median survival remains poor. However, whether this same approach can be used for primary brain tumors such as GBM, remains to be determined. Molecular analyses of patient samples suggest that the high TP:DPD ratio (the determinant of response to CAPE) in GBM compared with normal brain tissue would result in the preferential intratumor activation of CAPE. In addition, irradiation (a standard of care for the treatment of GBM) is shown to produce a tumor-associated induction of TP expression, which could result in improved CAPE efficacy. Most importantly, the induction of TP is shown to occur in distant (nonirradiated) tumors in the same animal. Although the combination of 5-FU plus radiotherapy has been shown to produce additive efficacy, preclinical studies in xenograft models suggest that the effect of CAPE plus radiotherapy is synergistic (Sawada et al., 1998; Sawada et al., 1999; Wilke, 2002). In addition, because 5-FU is a well-established radiosensitizing agent, potentially higher intratumoral levels of 5-FU (achieved by higher intratumoral TP levels) represent an opportunity to maximize the antitumor efficacy through the careful optimization of timing, dose, and administration of CAPE and irradiation. The pharmacogenomic

approach used in this study may provide the basis for the use of molecular markers in the rational design of new clinical treatment paradigms.

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CYTOKINE-MEDIATED STABILIZATION AND INCREASED TRANSCRIPTION OF THYMIDINE PHOSPHORYLASE (TP) MESSENGER RNA IN HUMAN GLIOMA CELLS: A POTENTIAL MECHANISM FOR INCREASED TP EXPRESSION AFTER GLIOMA XENOGRAFT IRRADIATION

by

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Abstract

Thymidine phosphorylase (TP) is the final and rate-limiting enzyme responsible for the conversion of 5'-deoxy-5-fluorouridine, a capecitabine (CAPE) metabolite, into the antitumor compound 5-fluorouracil. We recently reported a sustained increase of TP messenger RNA (mRNA) in U87MG glioma xenografts after irradiation (external beam radiotherapy [XRT]; Blanquicett, Gillespie, et al., 2002), suggesting the use of CAPE-XRT as a potentially synergistic chemoradiation regimen to treat gliomas. In this study, cytokine-mediated transcriptional and posttranscriptional effects on TP were examined using the U87MG and U251MG human glioma cell lines to elucidate to the mechanisms of TP up-regulation. Evaluation of 12 cytokines at 0-24 hr after XRT demonstrated increased expression of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1α mRNA in both U87MG and U251MG cells. Relative to control (phosphate-buffered saline [PBS]), a 24-hr incubation with 10 ng/ml of recombinant, human IFN- γ, TNF-α, IL-1α, or a combination of these 3 cytokines demonstrated increased TP mRNA levels by 4.5, 1.9, 1.2, and 7 fold in U87MG cells, respectively, and 7.3, 4.3, 2.0, and 11 fold in U251MG cells, respectively. CAPE (5'-deoxy-5-fluorouridine) cytotoxicity was then determined in U87MG and U251MG cells upon 5-day drug exposure in the presence or absence of 10 ng/ml IFN-γ, TNF-α, IL-1α, or a combination of these cytokines. Cells treated with either IFN- γ or the cytokine mixture were 100-fold more sensitive to CAPE at 0.01 ug/ml doses compared to PBS (control). TNF-α -treated cells demonstrated intermediate sensitivity to CAPE, with 10-fold less 5'-deoxy-5-fluorouridine (compared to PBS) required to reach an equivalent inhibitory concentration. IL-1α treatment did not significantly enhance CAPE cytotoxicity to either cell line. U87MG glioma cells transiently transfected with a cDNA encoding luciferase and driven by the human TP pro-

moter demonstrated a nearly 2-fold (>1.5) increase in luciferase expression upon treatment with TNF-α, IFN-γ, or the cytokine mixture, relative to control (PBS) and IL-1α (similar to controls). In U251MG cells, similar evaluation resulted in a 1.5-, 2.5-, and 2-fold increase in TP promoter activity after TNF-α, IFN-γ, and cytokine mixture incubation, respectively, and relative to PBS or IL-1α (no difference from PBS). Electrophoretic mobility shift assays demonstrated that the NF-kB, AP2/Sp1, and Sp1 regions of the TP promoter were potential, dose-dependent protein-binding sites for transcriptional activation. Furthermore, posttranscriptional effects were also examined in these cells. Results indicated that TNF-α increased TP mRNA half-life in both U87MG and U251MG cells by 2 and 3fold, respectively; that IL-1α treatment had no effect on mRNA half-life; and that IFN-γ treatment was similar to cytokine combination, increasing TP mRNA half-life by 3 and >3 fold in U87MG and U251MG cells, respectively. Finally, the contribution of the TP mRNA 3'untranslated region on the observed cytokine-mediated stabilization was determined in these cells by using a luciferase assay which demonstrated that the TP 3'UTR region, particularly the latter 130 nt (and not the 3' end region, containing the previously-hypothesized polypyrimidine-binding protein sequence), may significantly contribute to mRNA stability. These in vitro studies suggest potential cytokine-mediated mechanisms for the observed increase in TP mRNA expression after XRT of glioma xenografts. Insight into the mechanisms responsible for TP up-regulation may be used to improve the tumor-selective activation of CAPE, further supporting the rationale for clinical evaluation of CAPE-XRT chemoradiation in patients with malignant gliomas.

Introduction

Capecitabine (CAPE; N_4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) is an orally administered fluoropyrimidine prodrug that mimics continuously infused 5-fluorouracil (5-FU), which was rationally designed on the basis of the observed over-expression of the enzyme thymidine phosphorylase (TP) in different tumor types (Bajetta, Carnaghi, Somma, & Stampino, 1996; Ishikawa, Utoh, et al., 1998; Miwa et al., 1998). As the final and rate-limiting step catalyzing the conversion of 5'-deoxy-5-fluorouridine (5'-DFUR), a CAPE metabolite, into the active 5-FU compound, TP overexpression in a tumor may result in the preferential conversion of CAPE in that tumor (Pentheroudakis & Twelves, 2002). Although TP ultimately converts CAPE into 5-FU, dihydropyrimidine dehydrogenase is responsible for 5-FU degradation (Diasio, Beavers, & Carpenter, 1988). As a result of the delicate balance that exists between the activation and elimination of CAPE, previous studies have identified TP and dihydropyrimidine dehydrogenase as indicators of response to this drug (Ishikawa, Sekiguchi, Fukase, Sawada, & Ishitsuka, 1998; Mori et al., 2000). In particular, several pharmacogenomic studies have correlated the antitumor efficacy of CAPE with elevated intratumoral TP levels, which should theoretically, result in higher intratumoral levels of 5-FU (Ishikawa, Utoh, et al., 1998; Miwa et al.).

We recently reported a sustained increase in TP messenger RNA (mRNA) levels in GBM xenografts after external beam radiotherapy (XRT). This result supports the rationale for the use of CAPE and XRT in combined chemoradiation therapy of glioblastoma multiforme, a lethal disease with a dismal prognosis (Blanquicett, Gillespie, et al., 2002). Preclinical studies have demonstrated synergistic antitumor efficacy of CAPE-XRT chemoradiation, with the molecular basis for this synergy being attributed to an in-

duction of TP by XRT (Sawada, Ishikawa, Sekiguchi, Tanaka, & Ishitsuka, 1999). Therefore, XRT-mediated TP up-regulation may improve CAPE efficacy. However, the mechanisms for the regulation of TP have not been thoroughly studied; particularly in glioblastoma multiforme, a mechanism for the observed TP induction by XRT has not been characterized.

Cytokine induction after XRT has also been demonstrated. We hypothesize that XRT-induced cytokine expression may be responsible for the observed XRT-mediated TP up-regulation. Although the cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and interferon (IFN)-γ have been shown to induce TP in colorectal carcinoma cell lines (Eda et al., 1993), it is not known whether these factors exert similar effects in gliomas. Moreover, the potential mechanism(s) for TP up-regulation have not been clearly identified but may involve transcriptional or posttranscriptional events such as mRNA stabilization (Nabors et al., 2003).

In the current manuscript, we report the results of cellular studies initiated to investigate the role of cytokines in the XRT-induced, delayed, and prolonged increase in TP expression previously reported in glioma xenografts (Blanquicett, Gillespie, et al., 2002). In these studies, we specifically examined the effects of cytokines (TNF-α, IFN-γ, IL-1α, and a mixture or combination of these three) on TP mRNA transcription and stability. In addition, because TP up-regulation should result in greater cytotoxicity of CAPE, we determined the effects of recombinant cytokines on 5'-DFUR cytotoxicity in U87MG and U251MG human glioma cells in vitro. Potential transcription factor-binding sites on the TP promoter were also evaluated. Furthermore, we examined the effects of cytokines on the 3'untranslated region of the TP gene in potential cytokine mediated TP mRNA half-life stabilization. Taken collectively, these studies support the hypothesis that

XRT-induced up-regulation of TP in glioma xenografts is potentially cytokine-mediated and may identify possible strategies for optimizing and improving CAPE efficacy.

Materials and Methods

Cell Culture

Human glioma cell lines U87MG and U251MG (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium/F12 (Mediatech, Herndon, VA) containing 7% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) and 2 mM glutamine as previously described (Blanquicett, Gillespie, et al., 2002; Miller, Williams, Buchsbaum, & Gillespie, 2002). All cells were cultured at 37 °C in a 5% CO_2 atmosphere without antibiotics, were passaged less than 12 times during the course of these experiments, and were regularly checked for *mycoplasma* contamination using a polymerase chain reaction-based kit. Nearly confluent (75%) monolayers of cells were harvested using brief exposure to 0.05% trypsin/0.53 mM EDTA (Invitrogen Life Technologies, Carlsbad, CA). Harvested cells were pelleted (200 x *g*, 8 min, ambient temperature) in complete medium, washed with phosphate-buffered saline (PBS), and then respun. PBS was aspirated, and cell pellets were stored at -80 °C for subsequent use. 5'-DFUR was purchased from Sigma Chemicals (St. Louis, MO). Recombinant IL-1α, TNF-α, and IFN-γ were obtained from R & D Systems Inc. (Minneapolis, MN).

RNA Extraction

Total RNA was isolated as previously described (Blanquicett, Johnson, Heslin, & Diasio, 2002). All sample concentrations were calculated spectrophotometrically at ab-

sorbance at 260 nm and diluted to a final concentration of 20 ng/μl in RNase-free water containing 12.5 ng/μl of total yeast RNA (Ambion, Austin, TX) as a carrier.

Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR)

Expression levels were determined using an ABI 7700 Sequence Detection System as previously described by our laboratory (Blanquicett, Gillespie, et al., 2002; Johnson, Wang, Smith, Heslin, & Diasio, 2000). The primers and probes for human TP and S9 ribosomal RNA (GenBank Accession Nos. NM001953 and NM001013, respectively) were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The sequence and optimum primer/probe concentrations for S9 and TP have been previously described (Blanquicett, Gillespie, et al., 2002). Expression levels were calculated using the comparative C_t method as previously described (Blanquicett, Johnson, et al., 2002). All reactions were run in triplicate. Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template-control reactions were performed.

Cytokine Expression

The effect of irradiation on the expression of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN-γ, and TNF-α) in U87MG and U251MG cells was examined using a Taqman Cytokine Gene Expression Plate I (Applied Biosystems, Foster City, CA) according to manufacturer's instruction and as previously described (Blanquicett, Gillespie, et al., 2002). Nonirradiated glioma cells (controls) were used as the calibrator.

Effects of Recombinant Cytokines on TP mRNA Expression

U87MG and U251MG cells (0.5 x 10^6 per 25-cm^2 flask) were cultured for 24 hr. Cells were then incubated with 10 ng/ml of each individual cytokine (IL-1α, TNF-α, or IFN-γ), a mixture of the three cytokines, or PBS (vehicle) for 24 hr. Cells were harvested, total RNA was isolated, and TP mRNA was determined by Q-RT-PCR as described earlier.

The 5'-DFUR Cytotoxicity

Cytoxicity was evaluated using the Cyquant Cytotoxicity Assay as directed by the manufacturer (Molecular Probes, Eugene, OR). The IC_{50} value for 5'-DFUR was expressed as the concentration at which growth was inhibited by 50% compared to the control (PBS incubation), after a 5-day drug (5'-DFUR) exposure ranging in concentration from 0.0001 to 1,000 μg/ml.

TP Promoter Activity: Transient Transfection and Luciferase Reporter Gene Assay

U87MG and U251MG cells were cultured for 24 hr in 24-well format plates. Glioma cells were then transfected with both the construct containing the pGL3 luciferase reporter vector containing a full-length human TP promoter cDNA (pGL3-TP, 1 μg/well, provided by Dr. Vincenzo Guarcello) and the control vector phRL-thymidine kinase (0.5 μg/well), which was intended for use as an internal reporter or normalizer (Promega, Madison, WI). The second reporter is linked to a constitutive promoter (thymidine kinase) driving a low-level transcription that is unresponsive to the experimental factors used. The use of dual reporters enables the normalization of the experimental gene tran-

scription with respect to the control reporter transcription. Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 hr using conditions recommended by the manufacturer. After cytokine or PBS incubation, cells were lysed and assayed for TP and thymidine kinase promoter activity (luciferase) with a TD 20/20 luminometer (Turner Designs).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were purchased from Promega (Madison, WI) and stored at -80 °C. Gel Shift assays were performed according to the manufacturer (Promega, Madison, WI); however, nuclear extract, EDTA, glycerol, and poly(dI-dC) optimal concentrations (described below) were obtained by varying the concentrations of each of these components individually while maintaining the remainder component concentrations at a fixed level and running the assays. The 233-bp fragment of the TP promoter was end labeled with [γ-^{32}P]. The individual 30-bp double-stranded DNA oligonucleotides, representing fragments of the TP promoter sequence, are shown in Table 1, and were synthesized by Midland (Lubbock, TX) and gel purified by our laboratory. Oligonucleotides were used in excess (100X) and were included in the reactions as specific competitors for transcription factor-binding to the labeled probe. The radiolabeled probe was incubated with no nuclear extract (-) or with nuclear extract (2.5 μg of protein) and respective oligonucleotides in a final volume of 15 μl EMSA buffer (10 mM Tris, pH 7.5, 5% glycerol, 0.5 mM EDTA, pH 7.1, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mg/ml poly [dI-dC]) at room temperature for 20 min. For oligonucleotide competition analysis, a 10-, 100-, or 300-fold molar excess of cold competitor oligonucleotide was added to the mixture before adding the probe. The reaction mixtures were electrophoresed on a 4% poly-

Table 1

DNA Duplex Oligonucleotide Sequences Derived From the Human Thymidine Phosphorylase Promoter and Used as Competitors for EMSAs

Duplex oligonucleotide	Sequence
1(AP2) -169 to -142	5'-AGGGATCACCCCCCGCCGACGGCCCCGGG-3'
2(c-ET-s1/cREL/N-Myc) -138 to -108	5'-CGACGGCCCGGAACTTCCGCGTGTCCGGGGG-3'
3(NF-kB(+)/Ybox(-)/ccaatbox(-))-111 to -82	5'-GGGGCACCGGGGATTGGCCGGGGCGCGGC-3'
4(AP2/SP1) -78 to -49	5'-CAAGGCTTCCCGGGGGCGGCGACTGCCGAG-3'
5(SP1(-))-55 to -33	5'-TGCCGAGCTCCGCCCTCCAGGCG-3'
6(CG box (-)) -39 to -16	5'-CCAGGCGGCCCCACCCGCCTGCCG-3'
7(SP1 (-))-11 to +12	5'-GGGCGCCGCCGCCCCGCCGCCG-3'

Note. *Only the sense strand is shown, the (-) sign indicates the location of the consensus binding in the antisense strand.

acrylamide nondenaturing gel in 0.5X Tris-Borate-EDTA (45 mM Tris-Borate and 0.5 mM EDTA) at 4 °C and visualized by autoradiography.

TP mRNA Half-Life Determination After Cytokine Stimulation

mRNA half-life determinations were performed in U87MG and U251MG glioma cells as previously described (Nabors et al., 2003). Briefly, 1 x 10^6 cells were split into p 100s or p 60s (petri dishes) and incubated for 24 hr in Dulbecco's Modified Eagle Medium (Fetal Bovine Serum, 2.6 mM L-glutamine) to allow for adherence and 75% confluency. The medium was then changed to Dulbecco's Modified Eagle Medium + L-glutamine and no fetal bovine serum; a 24-hr adjustment was allowed. On the 3rd day, cytokine (IL-1α, TNF-α, IFN-γ, or a mixture of the three cytokines in 10 ng/ml medium) or sterile PBS (control) was added to the cells. Actinomycin-D in 10 μg/ml medium was added to the cells after a 24-hr incubation with cytokines or PBS. Next, harvesting took place at various time points (0, 2, 4, 6, 8, 10, and 12 hr after Actinomycin-D addition). Total RNA isolation followed for subsequent determination of TP mRNA half-life by RT-Q-PCR.

TP Protein and Activity Assays

A commercially available TP enzyme-linked immunosorbent assay was used as directed by the manufacturer (Roche Diagnostics, Nutley, NJ). DLD-1 cells (negligible-TP-expressing colorectal carcinoma cells) were included as a negative control, and SKBR-3 cells (high-TP-expressing breast carcinoma cells) served as the positive control. TP activity was determined by radioassay as previously described (el Kouni, el Kouni, & Naguib, 1993).

TP 3'UTR Constructs

The 3'UTR fragment of the TP gene, including the polypyrimidine-binding-protein region (PPBP) at the 3' end of the gene (245 nt) and a truncated 3'UTR (lacking the latter 130 nt), also containing the PPBP region (115 nt, 1530-1645), were amplified by polymerase chain reaction, after a first-strand synthesis reaction was performed with a first-strand synthesis kit as instructed by the manufacturer (Invitrogen, Carlsbad, CA). The following primers were used for the amplification reactions: forward 5'-AGGAGGCG-CTCGTACTCTC-3' and reverse 5'-GGCCGACACAAATTGGAG-3'. Nested polymerase chain reaction amplification followed with these primers: for the complete UTR, forward 5-AGGAGGCGCTCGTACT- CTC-3 and reverse 5'-CGCGTACGCGTGTG- CCCG-3; and truncated UTR: forward 5'-AGGA-GGCGCTCGTACTCTC-3' and reverse 5'GTTCGTGACTGTTCCAAAG-3'. The polymerase chain reaction products were run on a 1% agarose gel and purified using the Qiagen "Qiaquick" Gel-Extraction kit (Qiagen, Valencia, CA). The fragments were subcloned by a ligation reaction into a PSTBlue-1 vector (Novagen). Competent *Escherichia coli* cells were transformed and plated onto LB agar petri dishes, and plasmid purifica-

tions were done using the Qiagen Miniprep (Qiagen). Enzyme digestions after plasmid purifications were performed to confirm the correct products; this was further verified by sequencing (to ensure correct orientation of the insert). This insert was then subcloned into the pGL3 basic vector and evaluated for promoter activity using the dual luciferase assays as described earlier. The TP 3'UTR in the antisense orientation was used as the negative control.

Statistical Analysis

To evaluate differences between controls (PBS incubation) and cytokine treatment groups, ANOVA was performed. The value of alpha was set at 0.05.

Results

Cytokine Expression in U87MG and U251MG After XRT

Because several cytokines have been shown to increase after XRT, we evaluated the expression of 12 cytokines (including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN-γ, and TNF-α) in U87MG and U251MG cells at 0, 2, 8, and 24 hr post-XRT. Evaluation of cytokine expression after XRT demonstrated increased expression of IL-1α, IFN-γ, and TNF-α in both U87MG and U251MG cells (Table 2).

XRT induction of IL-1α was the greatest among the three cytokines in both cell lines, peaking at 24 hr by increases of nearly 3 and 7 fold in U87MG and U251MG, respectively. In U87MG, IFN-γ peaked at 24 hr with an approximate 2-fold increase, in comparison to a maximum increase of 4 fold (at 8 hr after XRT) in U251MG cells. TNF-α peaked at 24 hr in both cell lines with less than 2-fold increases in expression. Although

Table 2

Increased Messenger RNA Expression Levels of Cytokines After Irradiation (XRT) in U87MG and U251MG Glioma Cell Lines at 0, 2, 8, and 24 Hr After XRT

	IFN-γ		TNF-α		IL-1α	
Hr	U87MG	U251MG	U87MG	U251MG	U87MG	U251MG
0	0.0	0.00	0.00	0.98	0.00	0.00
2	0.4	0.00	0.00	0.84	0.00	0.00
8	1.6	4.16	1.44	1.09	1.24	2.22
24	2.2	2.34	1.87	1.91	2.97	6.92

Note. IFN-γ = interferon-γ; TNF-α = tumor necrosis factor-α; IL-1α = interleukin-1α.

IL-10 was previously observed to increase in xenografts after XRT, this was not the case in vitro. High mRNA expression of IL-8 was detected both in xenografts and in both glioma cell lines; however, during the time frame examined, an increase in expression was not observed after XRT. The remaining cytokines were below the limits of detection.

Increased Levels of TP mRNA in U87MG and U251MG After 24-hr Cytokine Incubation

To determine the effects of recombinant cytokines on TP expression in vitro, U87MG and U251MG cells were incubated with PBS (control), IL-1α, IFN-γ, TNF-α, or a combination of the three cytokines at a concentration of 10 ng/ml, for 24 hr. As shown in Table 3, of the three individual cytokines tested, IFN-γ had the greatest effect, with increased TP mRNA levels of approximately 5 and 7.3 fold in U87MG and U251MG cells, respectively. TNF-α increased TP mRNA in U87MG and U251MG cells by 1.9 and 4.3 fold, respectively. IL-1α induced TP the least with (1.2 fold in U87MG, and 2.0 fold in U251MG cells). However, a combination of all three cytokines yielded the greatest in crease in TP mRNA levels (7 and 11 fold in U87MG and U251MG cells, respectively).

Table 3

Thymidine Phosphorylase Messenger RNA Determination After 24-Hr Incubation of U87MG and U251MG Cells With Cytokine (10 ng/ml IFN-γ, TNF- α, IL-1α, or a Mix Of All Three) Compared to PBS

Cells	PBS	IL-1α	TNF-α	IFN-γ	Mix
U87MG	1	1.2 ± 0.26	1.9 ± 0.41	4.5 ± 0.28	6.9 ± 0.33
U251MG	1	2.0 ± 0.19	4.3 ± 0.33	7.3 ± 0.66	11 ± 1.57

Note. PBS = phosphate-buffered saline; IFN-γ = interferon-γ; TNF-α = tumor necrosis factor-α; IL-1α = interleukin-1α.

TP mRNA Correlation to TP Protein

TP mRNA levels (determined with Q-RT-PCR) correlated highly with both TP protein levels (analyzed by ELISA) and activity (determined by radioassays). The resulting correlation coefficients (r^2) were 0.93 and 0.74, respectively (data not shown).

Effects of Cytokines on CAPE (5'-DFUR) Cytotoxicity

As shown in Figure 1, the antiproliferative activity of 5'-DFUR (CAPE) was enhanced in U251MG cells with cytokine addition. 5'-DFUR cytotoxicity was determined in U251MG cells after a 5-day drug exposure with PBS incubation (control) or the addition of 10 ng/ml IFN- γ, TNF-α, IL-1α, or a mixture of cytokines. In cells treated with both IFN-γ and cells treated with a mixture of cytokines, IC_{50}s demonstrated 1-log (10-fold) lower doses relative to the cells treated with 5'-DFUR (PBS) alone; and at low doses of 5'-DFUR (0.1 μg/ml), both IFN-γ and the combined cytokines potentiated cytotoxicity by 100 fold (compared to PBS incubation). IL-1α incubation demonstrated cytotoxicity similar to that of PBS incubation. TNF-α demonstrated intermediate cytotoxicity relative to IFN-γ or mixture (highest cytotoxicity) and IL-1α or PBS/5'-DFUR (lowest

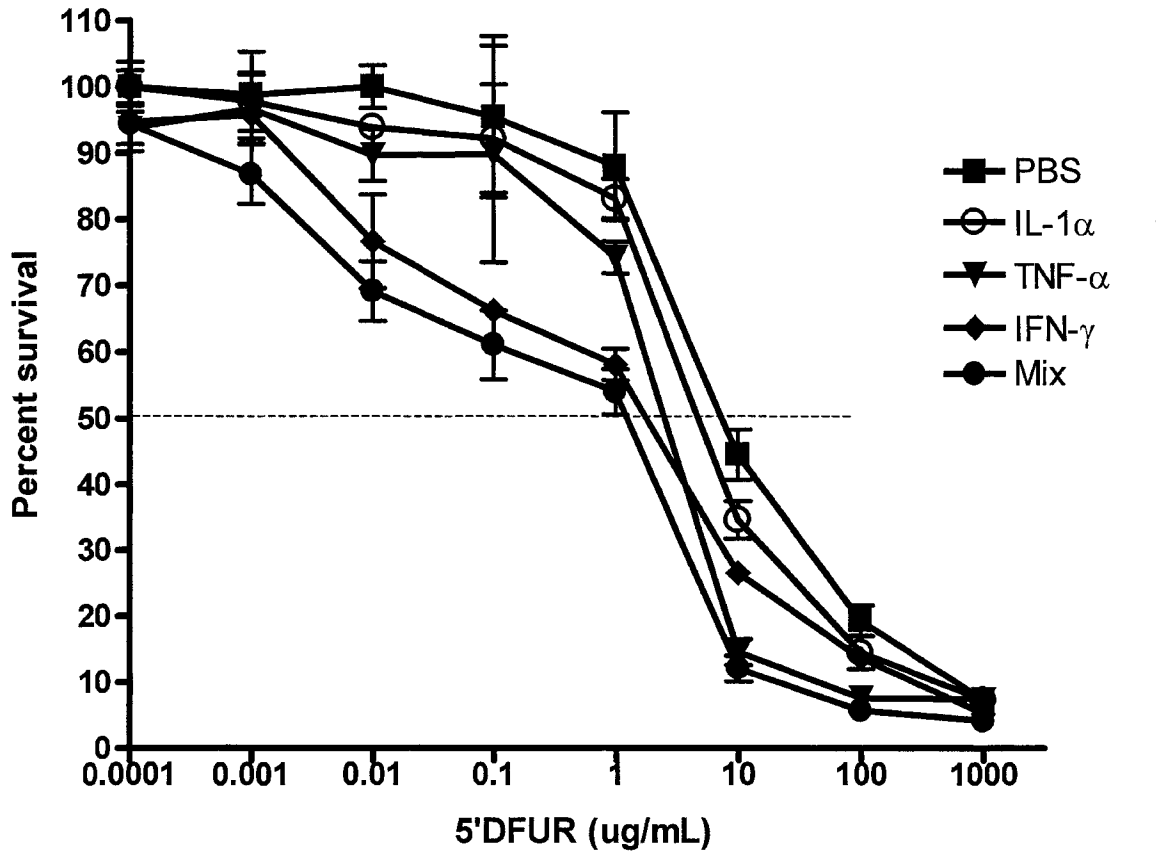

Figure 1. Cytotoxicity of 5'-deoxy-5-fluorouridine (5'-DFUR) in U251MG cells incubated with cytokines. U251MG glioma cells incubated with phosphate-buffered saline (PBS), interleukin (IL)-1α, tumor necrosis factor (TNF)-α, or interferon (IFN)-γ, or a mixture of these cytokins (Mix) were exposed to log doses of 5'-DFUR for 5 days, and cytotoxicity was determined as described in Materials and Methods. As shown, at 0.1-μg/ml doses of 5'-DFUR, PBS (control)- and IL-1α-incubated cells required 100-fold greater doses than IFN-γ- or cytokine-mix incubated cells did to achieve equivalent inhibitory concentrations. At 0.1 μg/ml 5'-DFUR, TNF-α-treated cells required 1-log lower doses to achieve the same inhibition that PBS-incubated glioma cells achieved.

cytotoxicity), with 5-fold less 5'-DFUR required to achieve an IC_{50} comparable to that of PBS incubation. The evaluation of U87MG cells demonstrated a pattern similar to that observed in U251MG (data not shown); however, U251MG cells were more sensitive (had a greater response) to 5'-DFUR and cytokines.

TP Promoter Activity After Cytokine Incubation

To examine the effects of recombinant cytokines on the TP promoter, TP-directed expression of luciferase activity was measured in U87MG and U251MG cells transiently transfected with the full-length TP promoter cDNA. As shown in Figure 2, IL-1α-incubated glioma cells (both U87MG and U251MG) did not demonstrate significant differences relative to PBS (control; $p > 0.05$). Conversely, IFN-γ- and TNF-α– incubated U87MG cells (Figure 2A) demonstrated significant increases in transcription, with approximately 2-fold higher levels in luciferase activity relative to controls ($p < 0.05$). The cytokine-mix incubation also demonstrated an approximately 2-fold increase; however, this increase was not significant in U87MG cells ($p > 0.05$). In U251MG cells (Figure 2B), TNF-α, IFN-γ, and the cytokine mix demonstrated significant ($p < 0.05$) increases in TP promoter activity, with approximately 1.5-, 2.5-, and 2-fold higher activities, respectively, compared to PBS (control). The modest changes in TP transcription observed after cytokine incubation (compared to increased mRNA levels as determined by RT-Q-PCR) suggested that other mechanisms were involved in the regulation of TP mRNA expression (e.g., message stabilization).

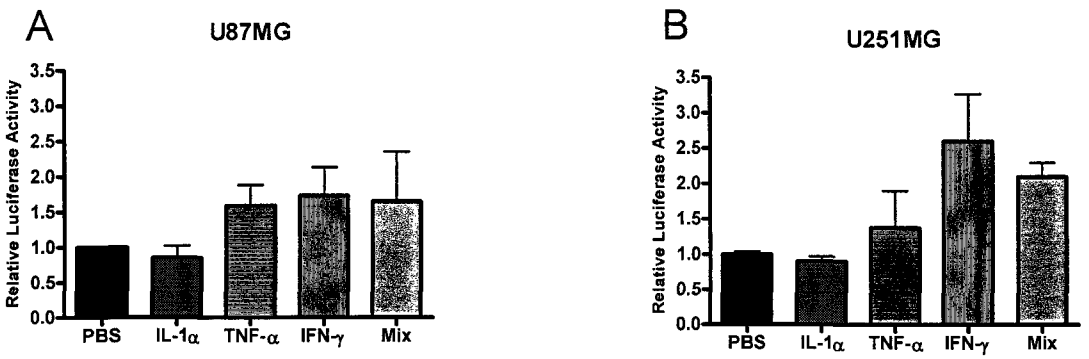

Figure 2. Thymidine phosphorylase promoter activity in U87MG and U251MG cells after cytokine incubation. A full-length thymidine phosphorylase promoter fragment was fused to the luciferase gene. Luciferase activity is expressed relative to the activity of phosphate-buffered saline (PBS) "stimulated" cells, with Renilla luciferase used as an internal control to normalize transfection efficiency. As shown for U87MG cells (A), nearly 2-fold differences were observed in thymidine phosphorylase promoter activity after tumor necrosis factor (TNF)-α, interferon (IFN)-γ, or cytokine-mixture (Mix) stimulation. In U251MG cells (B), TNF- α, IFN-γ and cytokine mix demonstrated significant increases in TP promoter activity, with approximately 1.5-, 2.5-, and 2-fold higher activities, respectively, compared to PBS incubation (control). Cells simulated with interleukin (IL)-1α had an intensity similar to that of PBS (control)-stimulated cells (both U87MG and U251MG).

Identification of the Specific TP Promoter-Binding Sites: EMSA

A candidate (Sp1) regulatory site for the TP promoter has been previously identified (Zhu, Lenzi, & Schwartz, 2002). To confirm this binding site and to determine other potential binding sites on the TP promoter, an EMSA was carried out (Figure 3) using a radiolabeled DNA 233-bp probe that spans the TP promoter elements. The DNA probe was incubated with (+) or without (-) partially purified HeLa extracts. As shown in Figure 3A, competition with various 30-mer-oligonucleotide probes (100-fold excess) containing known transcription factor-binding sequences (Table 1), revealed the NF-kB (Lane 3), AP2/Sp1 (Lane 4) and Sp1 (Lane 5) sites as potential binding sites, because these sites prevented transcription factor-binding to the labeled TP probe. In particular, the oligonucleotide containing the AP2/Sp1-binding site (Lane 4) completely prevented transcription factor-binding, appearing similar to negative controls (no HeLa; -). No or slight shifts in mobility (little binding) were observed in the remaining lanes, with Lane 6 (containing an imperfect GC box) remaining nearly identical to the positive control (+). Shown in Figure 3B are the results of dose-dependent competitive analyses (10X, 100X, or 300X excess of cold probes) of the potential NF-kB-, AP2/Sp1-, and SP1-binding sites. The results suggest an abrogation of transcription factor binding to the promoter DNA with increasing amounts of competitive probe, particularly for the AP2/Sp1-binding site (Lanes 6-8), for which just a 10-fold excess of cold probe (Lane 6) was sufficient to prevent transcription factor-binding to the radio labeled TP promoter probe.

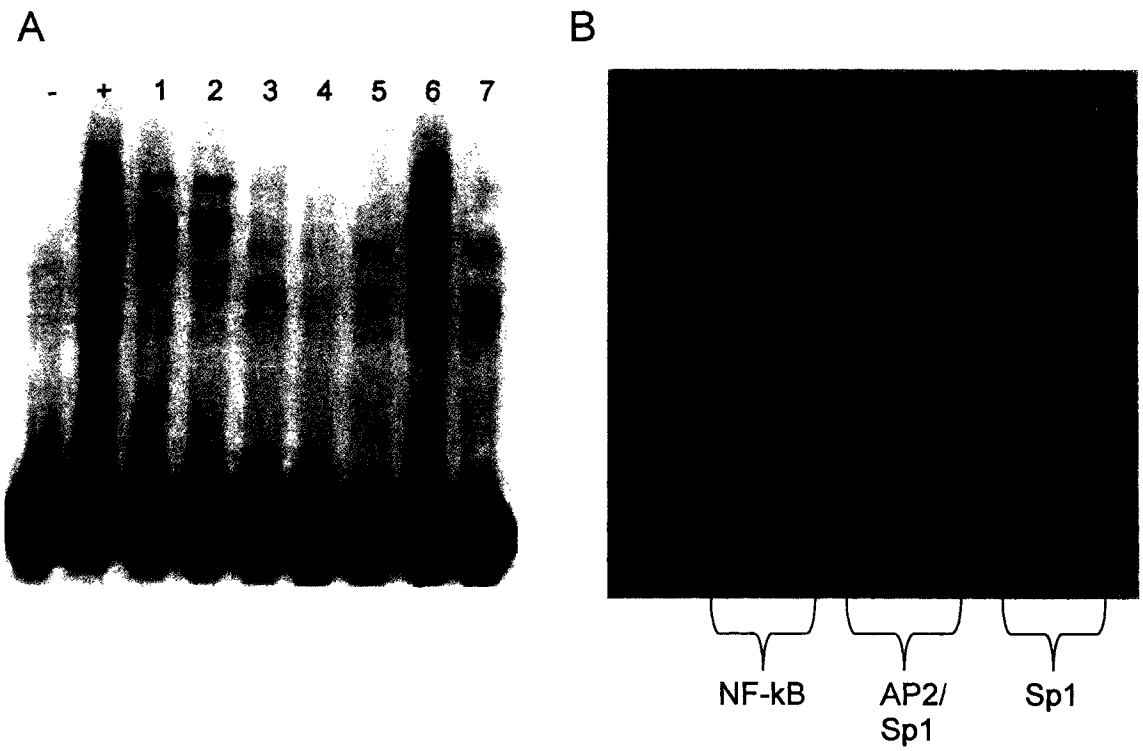

Figure 3. Electrophoretic mobility shift assay of the TP promoter. The 233-bp fragment of the TP promoter was end labeled with ^{32}P, and an analysis of specific interactions between transcription factors and the TP probe was performed. (A) double-stranded oligonucleotides containing transcription factor-binding sequences in the TP promoter for the NF-kB (lane 3), AP2/Sp1 (Lane 4), and Sp1 (Lane 5) sites prevented transcription factor-binding to the end-labeled TP probe. The AP2/Sp1-binding site (Lane 4) completely abrogated transcription factor-binding to the radiolabeled probe, appearing similar to negative (-) controls (no HeLa). Little binding was observed in the remaining lanes, with Lane 6 (containing an imperfect G/C box) being nearly identical to the positive (+) control. (B) Dose-dependent (10-, 100-, or 300X excess cold probes) competitive analyses of the NF-kB-, AP2/Sp1-, and Sp1-binding sites revealed transcription factor-binding competition with increased amounts of competitive probe, particularly for the AP2/Sp1-binding site (Lanes 6-8), for which just a 10-fold excess of cold probe (Lane 6) was sufficient to prevent transcription factor-binding to the probe.

Increase in TP mRNA Half-Life in Glioma Cells After Cytokine Incubation

The elevated levels of TP mRNA after cytokine incubation led us to investigate the contribution of posttranscriptional mechanisms to the up-regulation of TP mRNA. To determine any cytokine effects on TP RNA kinetics, U87MG and U251MG cells were incubated with one of the three cytokines of interest (IL-1α, TNF-α, or IFN-γ) or with a combination of these cytokines and compared to PBS-treated (control) cells. As shown in Figure 4, incubation with TNF-α (relative to PBS-incubated glioma cells) resulted in TP mRNA half-life increase of 2 and 3 fold in U87MG (Figure 4A) and U251MG cells, (Figure 4B), respectively. The RNA half-life was extrapolated from the decay curve at the time point representing 50% of the initial RNA levels immediately after Actinomycin D treatment. In Figure 5, incubation with of 10 ng/ml of IFN-γ resulted in an increment in TP mRNA half-life of 3 fold in U87MG (Figure 5A) and >3 fold in U251MG glioma cells (Figure 5B) relative to PBS incubation. These experiments were repeated at least three times, and representative experiments are shown (Figures 4 and 5). The effect of IL-1α incubation on TP mRNA half-life in these cells was similar to control (PBS incubation), and incubation with a mixture of cytokines demonstrated an increase in TP mRNA half-life similar to that found for incubation with IFN-γ alone (data not shown).

TP 3'UTR Construct Activity

To determine the effects of cytokines on TP 3'UTR activity, the ratio of TP to thymidine kinase (normalizer to determine transfection efficiency) luciferase activity was measured in U251MG cells transfected with the TP 3'UTR (245 nt), including the PPBP region at the 3' end. A schematic diagram of the luciferase plasmid is shown in Figure 6

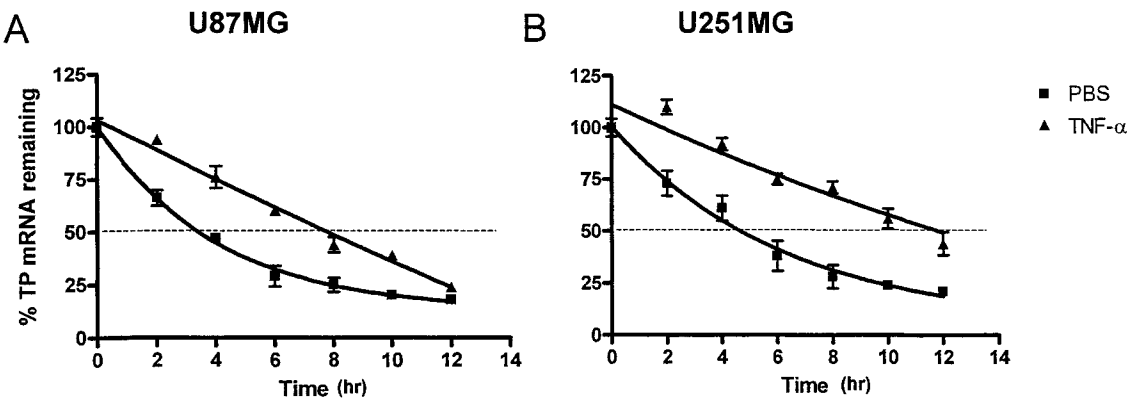

Figure 4. Thymidine phosphorylase (TP) messenger RNA (mRNA) half-life determination in U87MG and U251MG cells after tumor necrosis factor (TNF)-α incubation. (A) U87MG cells were incubated with 10 ng/ml TNF- α or phosphate-buffered saline (PBS; control) for 24 hr. Cells were harvested at 0, 1, 2, 4, 8, 10, and 12 hr after Actinomycin-D addition. TP mRNA was quantitated, and half-lives were calculated. TNF-α-incubated U87MG cells demonstrated an approximate increase of 2 fold in TP mRNA half-life relative to PBS-incubated cells. (B) A similar evaluation in U251MG cells demonstrated a 3-fold increase in TP mRNA half-life after TNF-α-incubation relative to PBS incubation.

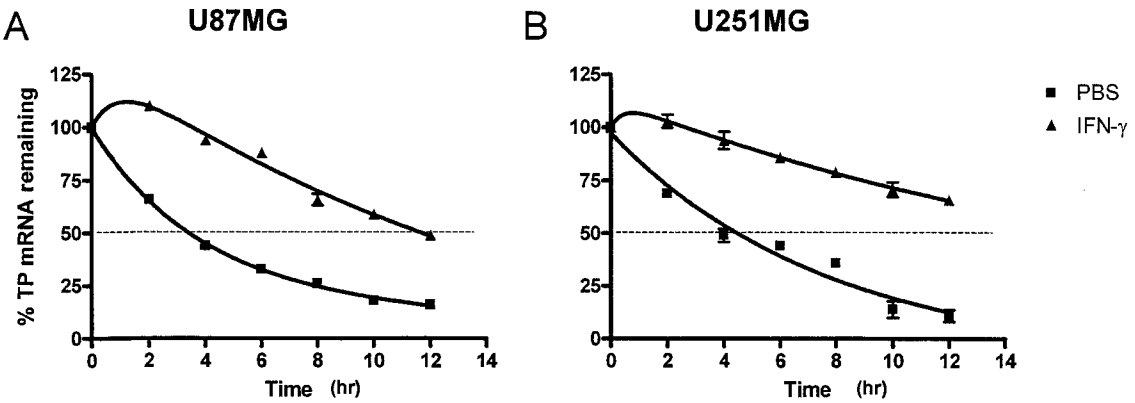

Figure 5. Thymidine phosphorylase (TP) messenger RNA (mRNA) half-life determination in U87MG and U251MG cells after interferon (IFN)-γ incubation. (A) TP mRNA half-life increased 3 fold in U87MG cells upon 24-hr incubation with 10 ng/ml IFN-γ. (B) In U251MG cells, TP mRNA half-life was increased by >3 fold after incubation with IFN-γ compared to incubation with phosphate-buffered saline (PBS; control).

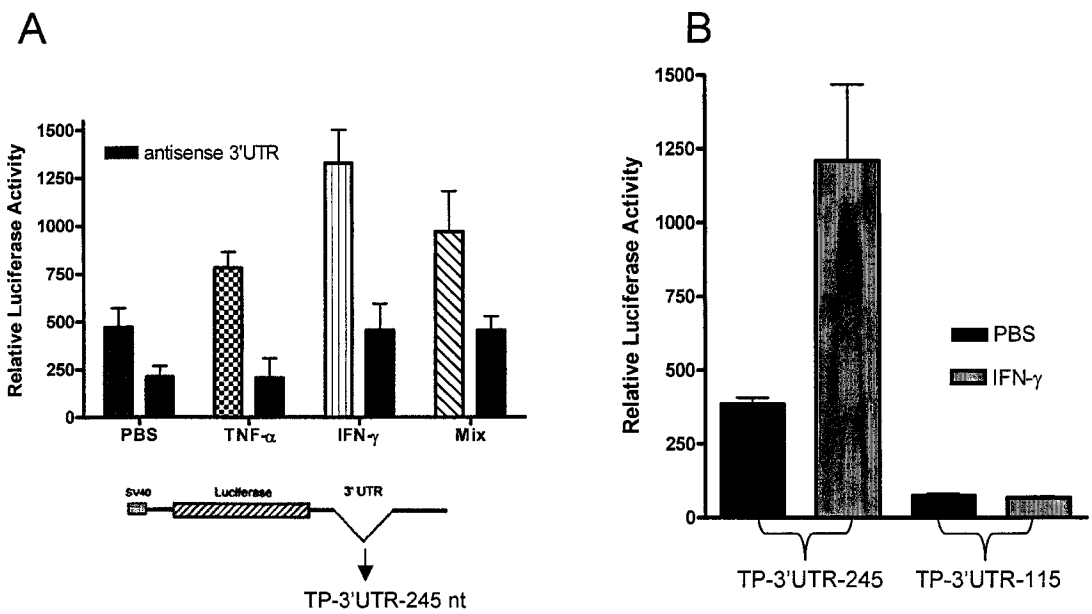

Figure 6. Luciferase activity in U251MG cells transfected with the thymidine phosphorylase (TP) 3'UTR and stimulated with cytokines. (A) Compared to incubation with phosphate-buffered saline (PBS; control), tumor necrosis factor (TNF)-α incubation increased luciferase activity by 1.7 fold, whereas interferon (IFN)-γ increased activity by nearly 3 fold (2.8). The cytokine-mix demonstrated an approximately 2-fold increase in luciferase activity. A schematic diagram of the luciferase plasmid is also shown (bottom). (B) Luciferase activity with the TP 3'UTR (TP-3'UTR-245) or the truncated 3'UTR (TP-3'UTR-115) was measured after IFN-γ or PBS incubation and was significantly reduced in the truncated 3'UTR.

A (bottom). Also shown in Figure 6A are the results of luciferase activity with cytokine or cytokine-mix incubation relative to PBS (control) incubation. Compared to control, TNF-α incubation increased activity by 1.7 fold, whereas IFN-γ increased luciferase activity by nearly 3 fold (2.8). Cytokine mix demonstrated an approximately 2-fold increase in luciferase activity. The antisense vector (TP 3'UTR in the negative orientation) was simultaneously evaluated as a reference. Because IFN-γ consistently demonstrated the greatest effects on TP, shown in Figure 6B is luciferase activity with the TP 3'UTR (TP-3'UTR-245) or the truncated 3'UTR (TP-3'UTR-115; also containing the PPBP region) after IFN-γ or PBS incubation. As demonstrated by results in the plasmid containing the truncated 3'UTR, deletion of the latter 130 nt of the TP 3'UTR significantly reduced luciferase activity despite the fact that the truncated 3'UTR contained the previously hypothesized PPBP region of potential stabilization.

Discussion

TP is the final enzymatic step in the conversion of the oral fluoropyrimidine, CAPE, into 5-FU. In human cancer xenograft models, CAPE given orally has yielded substantially higher concentrations of 5-FU within tumors demonstrating high expression of TP compared to normal tissue (Sawada et al., 1999). Even at equitoxic doses, 5-FU levels after CAPE administration have been shown to be much higher than those achieved by intravenous administration of 5-FU (Ishitsuka, 2000). This tumor-selective delivery of 5-FU (as a result of high tumor expression of TP) suggests greater efficacy and a safer profile of CAPE relative to other fluoropyrimidines. In addition, Ishitsuka contends that CAPE has characteristics not found with 5-FU, such as potent antimetastatic and anticachectic actions in mouse tumor models. Furthermore, the ability to orally

administer CAPE provides greater convenience in contrast to traditional fluoropyrimidine antineoplastics. Therefore, as a result of the advantages offered by CAPE, improving its efficacy and implementing it in novel treatment regimens may have substantial implications in the improvement of cancer treatment with fluoropyrimidines (Ishitsuka).

One such hypothesized method for improving CAPE efficacy involves up-regulation of TP (Sawada et al., 1998). We recently demonstrated elevated and prolonged levels of TP mRNA after XRT in glioma xenografts (Blanquicett, Gillespie, et al., 2002). These studies suggested a CAPE and concomitant XRT regimen for the treatment of gliomas; as a result, a Phase I clinical trial was initiated at our institution to examine CAPE chemoradiation for malignant gliomas (Newman et al., 2004). The mechanisms for the up-regulation of TP after XRT in both irradiated and contralateral glioma xenografts remain unknown; however, we previously suggested that these elevated levels were potentially caused by soluble factors such as cytokines, which have been shown to be induced after XRT and to up-regulate TP in colorectal carcinoma cells (Eda et al., 1993; Goto et al., 2001; Makower, Wadler, Haynes, & Schwartz, 1997). Despite these findings, the examination of the mechanisms for TP up-regulation still remains an area of active investigation, and these mechanisms have not been evaluated in glioma. A thorough understanding of the mechanisms responsible for TP up-regulation may be useful in the improvement of the therapeutic benefit of CAPE.

In the present study, we demonstrate an increase in TP mRNA in U87MG and U251MG glioma cells that were incubated with cytokines (IL-1α, TNF-α, IFN-γ, or a combination of the three). An increase in TP by cytokines should result in greater cytotoxicity of CAPE, which we demonstrate in glioma cells exposed to 5'-DFUR and incubated with cytokines. We suggest that this increase results from both transcriptional and

posttranscriptional (TP mRNA half-life increase) effects of cytokines on TP. We allude to the regions of potential transcription factor-binding sites on the TP promoter, and we also describe a correlation among TP mRNA, protein, and activity. Finally, we determine the importance of the 3'UTR in stabilizing TP mRNA by evaluating TP mRNA half-life in glioma cell lines after cytokine addition and by further examining the effect of cytokines on the activity of the TP 3'UTR.

XRT of glioma cells demonstrated increased mRNA expression levels of IL-1α, TNF-α, and IFN-γ. Although we previously observed an increase of IL-10 after tumor xenograft irradiation, this increase did not occur in cell culture. High mRNA expression of IL-8 was detected both previously in xenografts and currently in U87MG and U251MG glioma cell lines; however, an increase in expression was not observed after XRT during the 24-hr period examined. The evaluation of time points other than the specific time points assessed in this study may result in a cytokine expression profile different from that reported here. As a result of the cytokine profile observed in glioma cells and xenografts, we decided to focus on the effects of IL-1α, TNF-α, and IFN-γ (as well as a combination of these three cytokines) on TP expression. Incubation of glioma cells with these cytokines caused a marked up-regulation of TP mRNA levels. Although previous studies (Eda et al., 1993; Makower et al., 1997; Zhang, Mackenzie, Rees, & Bicknell, 1997) have reported this effect, glioma cell lines had not been evaluated before this study. Independently, IFN-γ caused the largest increase. Makower et al. have reported an in vivo induction of TP (mRNA and protein) by IFN (α and β), which was attributed to regulation at the mRNA level; however, regulation was not demonstrated. Goto et al. (2001) later showed that, of various inflammatory cytokines, IFN-γ most effectively increased TP expression in human macrophages and that this occurred through increased transcrip-

tion and not through increased stability of TP mRNA. Thus, we subsequently decided to investigate transcriptional effects of these cytokines on TP. However, transient transfection of glioma cells with the TP promoter, as demonstrated here, suggested that, because of a slight (2-fold) increase in transcriptional effects after incubation of U87MG cells with cytokine (and a maximum of 2.5 fold for U251MG cells incubated with IFN-γ), other mechanisms for TP up-regulation had to be involved. The involvement of other mechanisms was especially thought to be the case, because the increased amounts of TP mRNA after cytokine incubation surpassed 2-fold levels. It is possible that, in an isolated system that not isolated, such as in an in vivo one, cytokines may cause a cascade of other regulators that may activate transcription; however, as we demonstrate, a direct effect by cytokines on the TP promoter is not dramatic. Nevertheless, a transcriptional effect was demonstrated (especially by IFN-γ in U251MG cells, a finding in agreement with the results reported by Goto et al.), leading to our examination of potential promoter-binding sites through EMSA. Results revealed that the NF-kB-, AP2/Sp1-, and the Sp1-binding sites were potentially important because protein binding at these sites was demonstrated. Regarding the Sp1 site specifically, our data are in agreement with the findings of Zhu et al. (2002).

It is of particular interest that half-life stabilization effects by cytokines on TP mRNA were observed in this study. These effects were greater than the transcriptional effects were found to be, suggesting that the major contributing mechanism of TP mRNA increase is the extension of the TP mRNA half-life. In particular, IFN-γ had dramatic effects in half-life stabilization in U251MG cells. These data correspond with the higher TP mRNA levels demonstrated in these cells after cytokine addition (Table 2). These data further suggest that, because mRNA stabilization was not observed in human macro-

phages (Goto et al., 2001) and because greater stabilization was shown in U251MG compared to U87MG cells, the effect may be cell specific.

To validate the use of Q-RT-PCR in these studies, correlations among TP mRNA, protein levels, and activity were made. A high correlation between mRNA and activity indicates the utility of RT-Q-PCR in replacing enzyme activity assays in these studies. Furthermore, the predicted effect of greater cytotoxicity after TP mRNA up-regulation was demonstrated by our cytotoxicity assays, which showed an enhanced antiproliferative effect of 5'-DFUR after cytokine addition.

Although the 3'UTR would logically be considered a region for potential mRNA half-life prolongation, the 3' end of the gene may also be involved. Schwartz, Wan, Wang, and Baptiste (1998) have suggested that the pyrimidine-rich region located 40 nt from the transcription termination site of the mRNA may have a role in modulating TP mRNA stability. Therefore, we decided to examine the TP 3' end and the 3'UTR. This investigation revealed that the latter 130-nt region of the TP 3'UTR (and not the PPBP region at the 3'end) was significantly affected by IFN-γ (measured by luciferase activity). Future studies may allude to other factors binding to TP and regulating expression not only at the promoter site but in other regions of interest where there may be potential regulation.

Taken collectively, our data demonstrate two cytokine-mediated potential mechanisms (transcriptional and posttranscriptional) for TP up-regulation. These results suggest a possible explanation for our previously reported XRT-induced increase in TP expression in glioma xenografts, where this modulation is possibly occurring through cytokine-mediated transcriptional activation and stabilization of TP mRNA. This evidence may ultimately be used to improve the tumor-selective activation of CAPE. Furthermore, these

studies have implications in understanding and improving of the efficacy of chemoradiation regimens involving CAPE and may be useful in the treatment of gliomas.

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ANTITUMOR EFFICACY OF CAPECITABINE AND CELECOXIB IN IRRADIATED AND LEAD-SHIELDED, CONTRALATERAL HUMAN BxPC-3 PANCREATIC CANCER XENOGRAFTS: CLINICAL IMPLICATIONS OF ABSCOPAL EFFECTS IN A METASTATIC MODEL

by

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Abstract

External beam radiotherapy (XRT) remains one of the major modalities used to treat patients diagnosed as having locally advanced pancreatic adenocarcinoma. However, the effect of XRT on metastatic tumors outside the field of irradiation (abscopal effect) remains largely unknown. In the current study, we examined the effect of XRT alone and in combination with capecitabine and/or celecoxib in both irradiated and lead-shielded, contralateral BxPC-3 pancreatic cancer xenografts. This chemoradiation regimen was chosen on the basis of our molecular analysis of pancreatic adenocarcinoma.

Athymic mice were injected bilaterally with BxPC-3 cells, and treatment was initiated 28 days postimplant. During XRT (2 Gy for 5 consecutive days, administered on Days 0 and 24), one flank was irradiated while the rest of the body (including the contralateral tumor) was lead shielded. capecitabine (350 mg/kg) was administered on Days 0 through 13 and Days 24 through 37. Celecoxib (20 mg/kg) was incorporated in the mouse chow and administered throughout the study.

In irradiated xenografts, capecitabine and XRT demonstrated synergistic antitumor efficacy ($p = 0.008$), which was further improved with the addition of celecoxib ($p < 0.001$). In contralateral shielded xenografts, abscopal effects were observed. Whereas monotherapy with XRT demonstrated significant reduction in tumor area in irradiated xenografts, growth was promoted by 23% ($p < 0.001$) in contralateral lead-shielded tumors in the same animals relative to untreated tumors. Interestingly, synergistic antitumor efficacy occurred in these contralateral tumors when capecitabine was administered ($p < 0.001$), despite their being outside the irradiated field. The addition of celecoxib further inhibited tumor growth ($p < 0.001$). There were no significant changes in thymidine phosphorylase, dihydropyrimidine dehydrogenase, or cyclooxygenase-2 mRNA levels in

irradiated or lead-shielded tumors, suggesting that efficacy cannot be predicted solely from these previously identified indicators of response. Immunohistochemical examination of the proliferation marker Ki-67 demonstrated concordance with tumor response in both irradiated and contralateral shielded xenografts.

These results have implications in the rational design of treatment paradigms for pancreatic cancer where metastatic disease remains the primary cause of patient morbidity, and abscopal effects in tumors outside the field of irradiation may affect tumor response.

Introduction

Pancreatic cancer, the fourth leading cause of cancer mortality in the United States (Landis, Murray, Bolden, & Wingo, 1999; Sohn, 2002), is characterized by an unusual resistance to both radiation (external beam radiotherapy [XRT]) and chemotherapy. Despite highly aggressive therapeutic approaches, the overall median survival of 3-5 months and a 5-year survival rate of 0.4-3% have not appreciably changed in the last 80 years (Brasiuniene, Juozaityte, Brasiunas, & Inciura, 2003). Surgery remains the most effective treatment for pancreatic adenocarcinoma (PAC), the most common and malignant type of pancreatic cancer. However, only 10-15% of patients have tumors suitable for resection, and 30-70% of these patients will have local recurrences (Beger, Rau, Gansauge, Pock, & Link, 2003; Penberthy, Rich, & Adams, 2003). At the time of diagnosis, most patients have locally advanced or metastatic disease with involvement of the peritoneum, liver, lungs, or lymph nodes. Chemoradiotherapy with either 5-fluorouracil (5-FU; Moertel et al., 1981) or, more recently, gemcitabine (Czito, Willett, Clark, & Fernandez Del Castillo, 2000; Wolff et al., 2001) has become the most commonly employed treatment

modality. The current approach using XRT is to reduce the amount of toxicity to adjacent tissues by focusing treatment to the primary tumor area, the area of residual tumor, or the site of tumor excision (involved fields or intensity-modulated radiation therapy). However, the effect of localized XRT on metastatic tumors outside the irradiated field (abscopal effects), particularly in combination with chemotherapy, remains to be elucidated.

The term *abscopal* was first introduced by Mole in 1953 to describe the effects of localized XRT on distant tissue that is outside the field of radiation absorption. It should be clarified that this phenomenon does not refer to bystander effects mediated by gap-junction intracellular communication (Snyder, 2004) but refers to radiation responses that are seen in areas separate from the irradiated tissue and are mediated by the secretion of soluble factors from irradiated cells. Elucidation of the precise molecular components and mechanisms responsible for such abscopal effects remains an active area of investigation that is further complicated by conflicting reports of either proliferative or anti-tumor effects in cells outside the field of irradiation (Camphausen et al., 2001; Demaria et al., 2004; Nobler, 1969; Ohba et al., 1998; Ohuchida et al., 2004; Qian et al., 2003). Although antiproliferative abscopal effects have been attributed to circulating lymphocytes, cytokines, or immune mediators, proliferative effects have been suggested to occur via activation of matrix metalloproteinases and growth factors (Camphausen et al; Demaria et al.; Nobler; Ohuchida et al.; Qian et al., 2002; Qian et al., 2003). In addition, although abscopal effects have been reported in a variety of malignancies including lymphoma, papillary adenocarcinoma, melanoma, adenocarcinoma of the esophagus, chronic lymphocytic leukemia, and hepatocellular carcinoma, there have been surprisingly few studies in advanced PAC, where chemoradiotherapy is commonly employed and where progression of metastatic disease remains the primary cause of morbidity.

Previous studies by members of our laboratory examining the correlation between drug-metabolizing enzymes and the potential efficacy of fluoropyrimidine chemotherapy in combination with radiation therapy demonstrated abscopal effects in contralateral lead-shielded xenografts (Blanquicett, Gillespie, et al., 2002). These studies, combined with tumor tissue analysis suggesting response to capecitabine (CAPE) on the basis of the expression of the indicators of response, thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD), provided the rationale for two Phase I clinical trials at our institution; in these trials, we examined the concurrent administration of CAPE and XRT for the treatment of both glioblastoma multiforme and PAC (Newman et al., 2004; Saif et al., 2004).

In the current study, we examined the effect of XRT alone and in combination with CAPE and/or celecoxib in both irradiated and lead-shielded, contralateral BxPC-3 xenografts. This model, consisting of animals containing both an irradiated tumor and a distant tumor outside the field of irradiation, was used to represent metastatic disease. The addition of celecoxib was chosen on the basis of our preliminary molecular analysis of surgically resected PAC biopsies demonstrating cyclooxygenase-2 (COX-2) overexpression, a marker of tumor progression and invasion. Taken collectively with previous studies demonstrating the abscopal effects of XRT, the radiosensitizing properties of 5-FU in pancreatic cancer (Fung et al., 2003), a selective increase in TP levels after XRT (Blanquicett, Gillespie, et al., 2002; Sawada, Ishikawa, Sekiguchi, Tanaka, & Ishitsuka, 1999), and the potential benefits of COX-2 inhibition (Crane, Mason, Janjan, & Milas, 2003), our examination of this trimodal regimen may be potentially useful in establishing future treatment paradigms for pancreatic cancer.

Materials and Methods

Tissue Preparation

After a protocol approved by the Institutional Review Board for Human Use, we obtained primary pancreatic ductal adenocarcinoma ($n = 5$) and uninvolved (normal) pancreatic tissues ($n = 5$) from cancer patients undergoing surgical resection. Tissues to be utilized for RNA extraction were snap frozen in liquid nitrogen and stored at -80 °C. Before RNA extraction, a 5-μm section was obtained from frozen tissue that had been fixed and paraffin embedded; it was then stained with hematoxylin and eosin so that it could be examined by a pathologist to confirm a diagnosis.

RNA Extraction

Total RNA was isolated using the Qiagen RNA Purification kit according to manufacturer's instructions (Qiagen, Valencia, CA). All sample concentrations were determined spectrophotometrically at A_{260} and diluted to a final concentration of 20 ng/μl in RNase-free water containing 12.5 ng/μl of total yeast RNA (Ambion, Austin, TX) as a carrier.

Quantitative Real-Time Polymerase Chain Reaction

Expression levels were determined using an ABI 7900 Sequence Detection System as previously described by members of our laboratory (Blanquicett, Johnson, Heslin, & Diasio, 2002; Johnson, Wang, Smith, Heslin, & Diasio, 2000). The real-time quantitative PCR primers were as follows: human TP forward (5' TCC TGC GGA CGG AAT CC-3'), reverse (5'-TGA GAA TGG AGG CTG TGA TGA G-3'), and fluorophore-labeled probe (FAM-CAG CCA GAG ATG TGA CAG CCA CCG T–TAMRA); COX-2

forward (5'-GAA TCA TTC ACC AGG CAA ATT G-3'), reverse (5'-TCT GTA CTG CGG GTG GAA CA-3'), and probe (FAM-TGG CAG GGT TGC TGG TGG TAG GA-TAMRA). The sequence for the primers and probes for human DPD and S9 ribosomal subunit (S9) have been previously described (Blanquicett, Gillespie, et al., 2002; Johnson et al., 2000). Expression levels were calculated using the relative standard curve method (Blanquicett, Johnson, et al., 2002; Johnson et al., 2000). All reactions were run in triplicate, and standard curves with correlation coefficients falling below 0.98 were repeated. Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template-control reactions were performed.

Immunohistochemistry (IHC)

TP protein levels were evaluated in PAC, uninvolved pancreas, colorectal carcinoma (CRC), and normal mucosa by IHC using the Anti-Thymidine Phosphorylase Antibody, Formalin-Grade kits according to the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, 5-μm sections were deparaffinized and rehydrated before undergoing antigen retrieval by steaming for 5 min. Sections were blocked with 20% normal goat serum for 20 min before overnight incubation at 4 °C with TP antibody/antiserum at a dilution of 1:100. Secondary Envision+ peroxidase antibody (Dako Incorporated, Glostrup, Denmark), specifically antimouse for TP, was added to the sections for 30 min before color development with liquid diaminobenzidine tetrachloride (Dako Incorporated, Glostrup, Denmark) for 20 min and counterstaining with Mayer's haematoxylin. After each incubation step, sections were washed twice with 1X phosphate- buffered saline for 5 min. A negative control section without the addition of TP-

specific antibody was included for each case. Immunostains were assessed on the basis of their intensity.

Ki-67 IHC in BxPC-3 xenografts on Day 50. We determined the proliferation index of both irradiated and shielded xenograft samples immunohistochemically by analyzing the expression of Ki-67 as previously described (Grizzle, Myers, Manne, & Srivastava, 1998; Manne et al., 1997; Porschen, Lohe, Hengels, & Borchard, 1989). Briefly, 5-μm-thick tissue sections were obtained from formalin fixed paraffin embedded tissue and placed on SuperFrost/Plus slides (Fisher Scientific, Norcross, GA), deparaffinized in xylene, and subsequently rehydrated in graded ethanols. Antigen retrieval was then performed by steaming for 5 min in a pH 8, 0.01 M EDTA solution. The sections were then transferred to a Tris-buffer bath (0.05 M Tris base, 0.15 M NaCl, 0.01% Triton X-100, pH 7.6). Each section was treated with an aqueous solution of 3% H_2O_2 for 5 min in order to quench endogenous peroxidase activity. Sections were then incubated with 3% goat serum at room temperature for 20 min to reduce nonspecific immunostaining. The primary antibody used was an anti-Ki-67 rabbit monoclonal antibody (clone: SP6, Lab-Vision Co., Fermont, CA; dilution: 1:400). Negative controls were performed by omitting the primary antibodies. Sections from tonsils served as positive controls. Secondary detection was accomplished using the USA-Ultra Streptavidin Detection System (Signet Inc., Dedham, MA). The sections were exposed to a biotinylated antirabbit antibody for 20 min, and a peroxidase-conjugated streptavidin was added for 20 min. A diaminobenzidine tetrachloride supersensitive substrate kit (Dako Inc.) was used to visualize the antibody-antigen complex. Each section was then counterstained using hematoxylin, dehydrated using graded alcohols, and soaked in xylene prior to coverslipping. The slides

were examined by a pathologist (D.C.C.). Positive staining was defined as the presence of nuclear staining regardless of intensity. The proportion of tumor cells that demonstrated nuclear staining was estimated as a percentage of total tumor cells.

Cell Culture

BxPC-3 pancreatic carcinoma cells (purchased from the American Type Culture Collection, Manassas, VA) were maintained in stationary monolayer cultures at 37 °C and 5% CO_2 in a humidified atmosphere using RPMI supplemented with 10% heat-inactivated fetal bovine serum and 2.6 mM L-glutamine. The BxPC-3 cell line was used because of its high expression of COX-2 (El-Rayes, Ali, Sarkar, & Philip, 2004). All cell cultures were maintained in antibiotic-free conditions and regularly checked for *mycoplasma* contamination using a polymerase chain reaction-based kit (American Type Culture Collection, Manassas, VA). Nearly confluent (75%) monolayers of cells were harvested by brief exposure to 0.05% trypsin/0.53 mM EDTA (Life Technologies, Gaithersburg, MD). Harvested cells were pelleted (200 x *g*, 8 min at ambient temperature) in complete medium and resuspended in serum-free medium. Viable cells were counted with a Neubauer hemacytometer and trypan blue (0.4%) exclusion.

Pancreatic Cancer Xenograft Preparation and Irradiation

Athymic, nude NCr mice (nu/nu) were subcutaneously injected bilaterally into hind flanks with a suspension of 1 x 10^7 BxPC-3 pancreatic cancer cells. Tumors were allowed to develop to 35-40 mm^2 in size (28 days postinjection). Tumor surface area was determined with vernier calipers by multiplying the length of the tumors by their width, and tumor growth was monitored twice per week. Mice were randomized into control and

treatment groups, with each group containing 12-15 mice. One of the pancreatic tumor-bearing flanks of the treated group was irradiated while the rest of the mouse (including the liver and the tumor in the contralateral flank) was lead shielded. XRT was carried out using a ^{60}Co teletherapy X-ray unit (Picker, Cleveland, OH) and was administered (2 Gy for 5 consecutive days) on Days 0 (start of treatment day, which occurred 28 days after cell injection) and 24. Mice in control groups were anesthetized with ketamine/zyloxine but were not irradiated. CAPE (Xeloda, Hoffmann-La Roche, Inc., Nutley, NJ) was dissolved in 40 mM citrate buffer (pH 6.0)/5% gum arabic and administered by gavage at a dose of 350 mg/kg for 14 consecutive days (Days 0-13), followed by 10 days of rest before administration of the second, 14-day treatment cycle (Days 24-37). Celecoxib (Celebrex, Pfizer) was incorporated in the mouse chow (20 mg/kg/day) and continuously administered throughout the duration of the 50-day study. Mice were sacrificed at various time points (0, 4, 12, 24, 32, 45, and 50 days) throughout the study for tissue analysis (gene expression and IHC).

Statistical Analysis

To evaluate differences in gene expression between normal and tumor tissues, paired *t* tests were performed where alpha was set at 0.05. The tumor growth curve data were analyzed by a log-linear mixed model approach on repeated measures ANOVA, using the MIXED procedure in SASR (Littell, Stroup, & Wolfinger, 1996). To determine whether any combination therapy arms produced significant synergistic inhibition of tumor growth (i.e., more than additive), the tumor growth curves from the serial area (log) measurements were compared using a two-way repeated measures analysis. To test for synergistic effects of the combination therapies, an interaction term was included in the

model. If the interaction term was significant and if the effect was inhibition of growth at a rate greater than additive, then the interaction was considered synergistic. The mean tumor-tripling times were analyzed using the Kruskal-Wallis test, and Fisher's exact test was used to compare the proportions of tumor size decrease.

Results

Quantitation of TP, DPD, and COX-2 Expression in PAC and Normal Pancreatic Tissues

As shown in Figure 1, TP expression is approximately 7.5-fold higher in PAC (mean = 41.7; *SE* = 8) than in normal pancreatic tissue (mean = 5.5; *SE* = 1.5), with a mean difference of 36.2. These differences were statistically significant ($p < 0.05$). There was no statistically significant difference in DPD levels between normal (mean = 12.5; *SE* = 5) and tumor (mean =10; *SE* = 3.5) tissue samples; the mean difference was 2.6 ($p > 0.05$). The average TP/DPD ratio demonstrated in PAC (4.2) is approximately 9.4-fold higher than that of normal pancreatic tissue (0.4). The higher ratio in PAC was primarily cause by higher expression of TP compared to normal pancreas ($p < 0.05$). COX-2 messenger RNA (mRNA) levels in PAC (mean = 22.2; *SE* = 11.9) were >100-fold higher compared to normal pancreas (mean = 0.2; *SE* = 0.1), with a mean difference of 22 ($p < 0.05$). Greater variability in COX-2 expression in tumor tissue relative to normal tissue was also observed.

IHC of PAC, Normal Pancreas, CRC, and Normal Mucosa

To determine whether the increased TP mRNA levels in PAC correlated with protein levels, IHC was done in PAC and uninvolved pancreas, as well as in CRC and

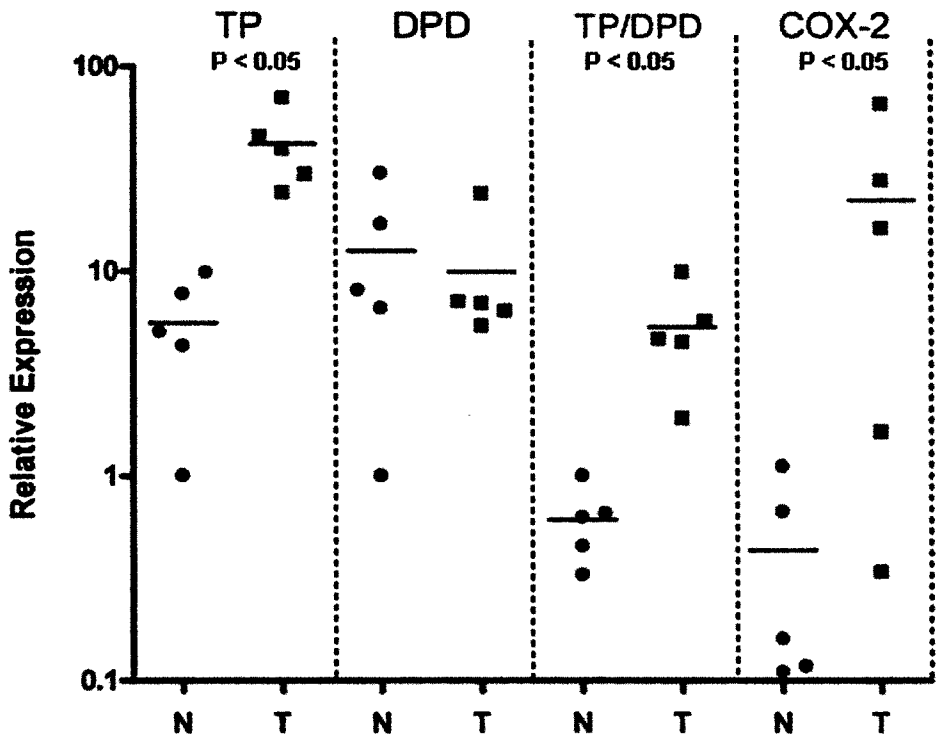

Figure 1. Expression of thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), and cyclooxygenase-2 (COX-2) messenger RNA in normal pancreatic tissue (N) and pancreatic adenocarcinoma (PAC; T). TP expression was 7.5-fold higher in PAC (■) than in normal pancreas (●). There was no significant difference in DPD expression levels in the same tissue samples. The average TP/DPD ratio demonstrated in PAC is approximately 9.4-fold higher than that of normal pancreas and is primarily caused by TP overexpression. This profile should result in selective conversion of capecitabine (CAPE; into 5-FU) in tumor compared to normal pancreatic tissues. COX-2 expression is over 100-fold higher in PAC than in normal pancreatic tissue.

normal mucosa as reference. As shown, TP staining in uninvolved pancreatic tissue (Figure 2A) demonstrated a few areas of faint staining, predominantly in the cytoplasm of the acinic cells and focally in the cytoplasm of the ductal cells. Scattered staining of TP can also be observed in the surrounding stroma. In Figure 2B (PAC), however, strong TP-specific immunoreactivity is observed in the neoplastic ducts of a well differentiated pancreatic ductal carcinoma. Although stromal cells also demonstrated faint and scattered staining of TP, in PAC (Figure 2B), intense and diffuse cytoplasmic staining is observed in the ductal adenocarcinoma cells. Figure 2C demonstrates TP staining in normal mucosa relative to CRC (Figure 2D). In the normal mucosa (Figure 2C), TP expression is noted predominantly in the stroma. In contrast to the staining seen in pancreatic cancer tissue, no staining is noted in the colonic crypts here. Figure 2D demonstrates TP staining in CRC. Unlike PAC (Figure 2B), several GI malignancies such as CRC (Figure 2D) demonstrate TP expression that is predominantly localized to the stroma. In addition, CRC demonstrates very weak cytoplasmic staining in the neoplastic glands (TP stain, x 100 for all samples).

BxPC-3 Tumor Xenografts

As illustrated in Figure 3, athymic NCr mice were subcutaneously injected with BxPC-3 pancreatic cancer cells in both hind flanks and allowed to develop tumors. To represent metastatic disease, one of the tumor-bearing flanks of the treated groups was irradiated, whereas the rest of the mouse (including the contralateral tumor) was lead shielded. Shown in Figure 4 are the effects of treatment with CAPE, XRT, and/or celecoxib alone and in combination on the tumor surface area in both irradiated (Figure 4A) and lead-shielded, contralateral xenografts (Figure 4B).

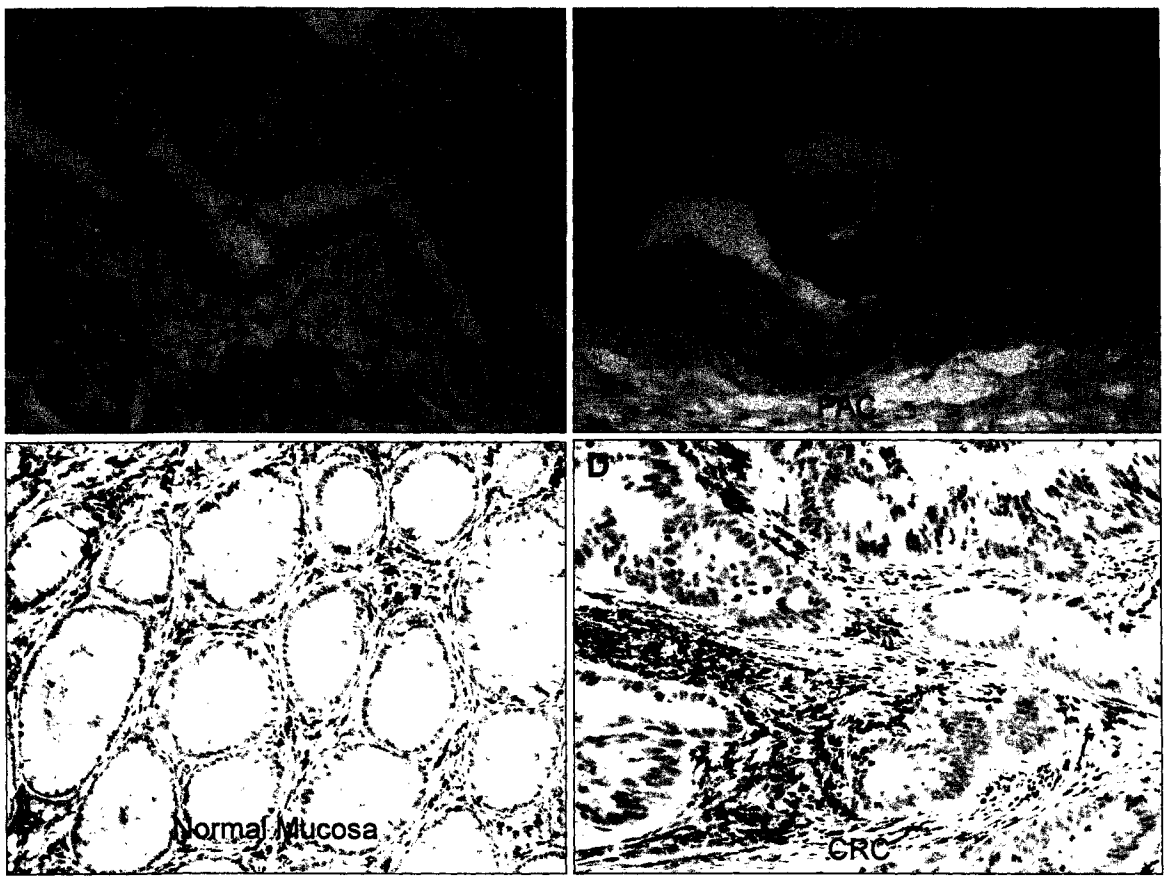

Figure 2. Immunohistochemical localization of thymidine phosphorylase (TP) in pancreatic adenocarcinoma (PAC), colonic carcinoma (CRC), and corresponding normal tissues. (A) Normal pancreas tissue. TP expression occurred predominantly in the cytoplasm of the acinic and ductal cells and was scattered staining in the surrounding stroma. (B) Pancreatic ductal carcinoma (PAC). Diffuse and intense cytoplasmic TP staining was observed in the neoplastic ducts. Scattered staining of TP was also noted in the surrounding stroma. (C) TP staining in normal colonic mucosa. TP expression was noted predominantly in the stroma. In contrast to pancreatic tissue, the colonic crypts showed no staining. (D) CRC tissue. Intense TP expression was noted in the surrounding stroma, with very weak cytoplasmic staining seen in the neoplastic glands.

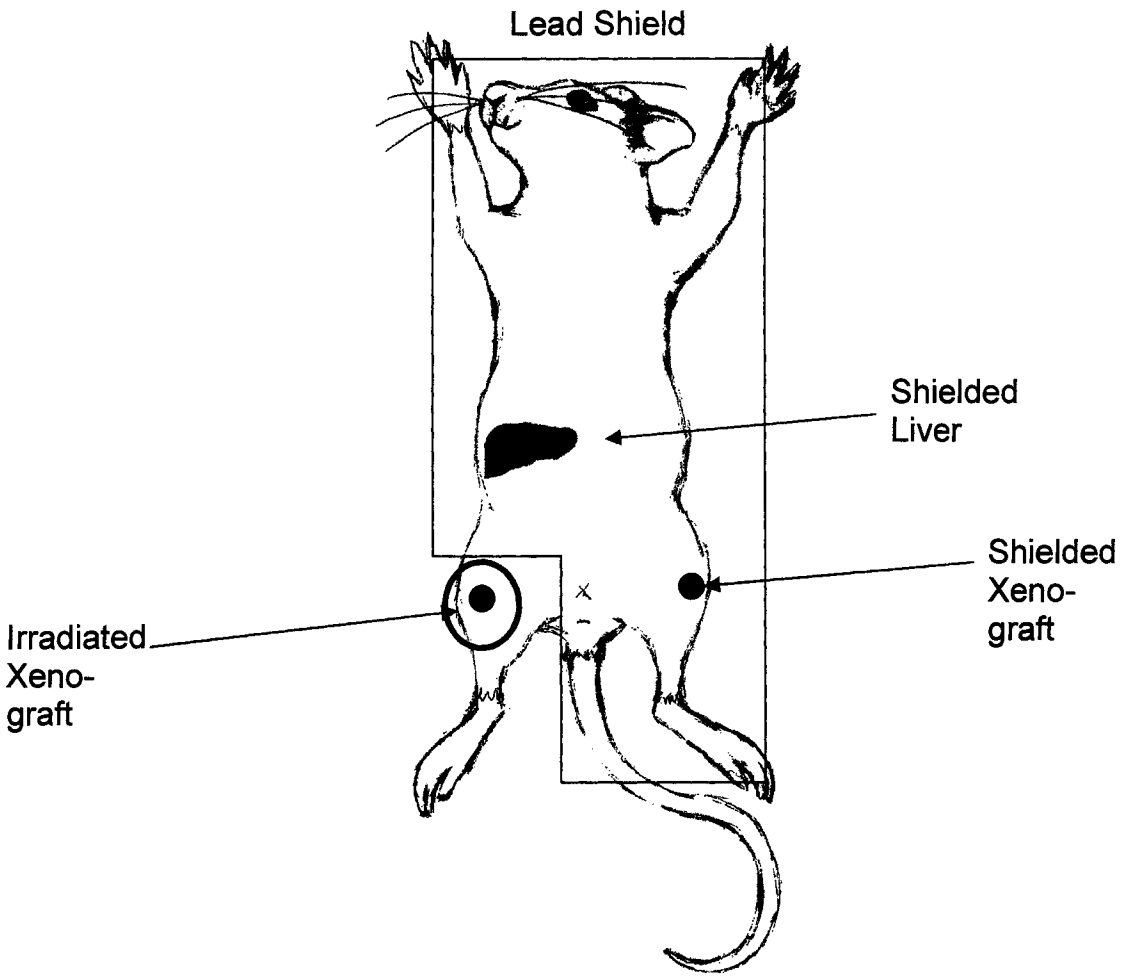

Figure 3. Schematic of mouse xenograft location(s) and irradiation. One of the two flanks containing a BxPC-3 xenograft was irradiated (circled), whereas the contralateral flank (containing the other BxPC-3 xenograft) and the body of the mouse were lead shielded.

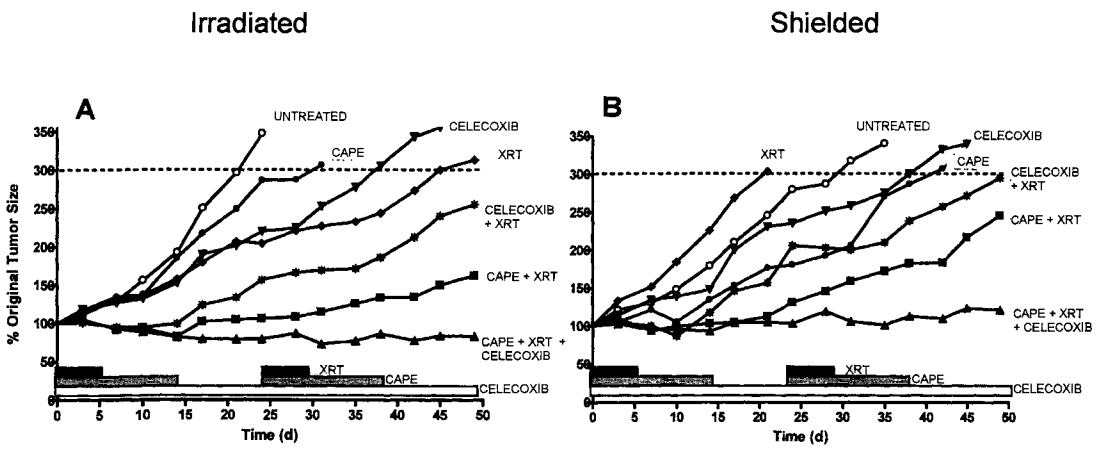

Figure 4. Percentage of change in tumor surface area versus time in both irradiated (A) and shielded, contralateral (B) BxPC-3 xenografts. Also depicted is the treatment schedule throughout the duration of study (external beam radiptherapy [XRT] ▬, capecitabine [CAPE] ▭, celecoxib ═). (A) In irradiated xenografts among CAPE, celecoxib, or XRT monotherapy, only treatment with XRT produced a significant reduction in tumor area ($p < 0.05$). The combination of CAPE and fractionated XRT demonstrated antitumor synergy ($p = 0.008$). The trimodal combination (CAPE-XRT, and celecoxib) was the most effective regimen, maximally decreasing tumor area by 27% ($p < 0.001$). (B) Proliferative abscopal effects of XRT promoted growth by 23% in contralateral lead-shielded tumors within the same animals. The surface areas of tumors treated with monotherapy with CAPE or celecoxib were not significantly different from those of untreated tumors ($p > 0.05$). The celecoxib and contralateral XRT combination did not produce a significant antitumor effect ($p > 0.05$). However, shielded xenografts demonstrated antitumor synergy with CAPE and contralateral XRT ($p < 0.001$). Similar to irradiated xenografts, shielded xenographs were most responsive to the trimodal combination despite these tumors' being outside the field of irradiation ($p < 0.001$).

Irradiated tumors. As shown in Figure 4A, untreated tumors demonstrated tripling times of approximately 20 days. In these xenografts, monotherapy with XRT was the most effective treatment, followed by celecoxib and CAPE, with tripling times of 44, 36, and 28 days, respectively. However, only XRT achieved statistically significant differences from untreated controls ($p < 0.05$). The combination of celecoxib and XRT produced an additive and statistically significant inhibition of tumor growth ($p < 0.05$). However, the combination of CAPE and XRT produced a synergistic inhibition of tumor growth ($p = 0$ .008) that was further improved with the addition of celecoxib ($p < 0.001$). As shown in Figure 4A, combination (both dual and trimodal) therapy prevented tumors from tripling; therefore, tumor surface area was used to determine response. The celecoxib-XRT and the CAPE-XRT combinations produced maximal reductions in tumor area (set at 100% at the start of treatment) to 94 and 82%, respectively. However, the trimodal combination of XRT-CAPE-celecoxib was the most effective regimen (Figure 4A), maximally decreasing tumor area to 73% of the original size ($p < 0.001$).

Contralateral, lead-shielded tumors (abscopal effects). In this model, lead-shielded tumors were also evaluated to determine whether abscopal effects of XRT occurred. As shown in Figure 4B, untreated contralateral tumors demonstrated tripling times of approximately 27 days. The differences in tripling times between untreated tumors (20 days on one side vs. 27 days on the contralateral side) were not statistically significant ($p > 0.05$). Monotherapy with either celecoxib or CAPE did not demonstrate significant differences from untreated tumors ($p > 0.05$). Independently, CAPE and celecoxib demonstrated tripling times of 41 and 36 days, respectively, compared to 27 days for untreated tumors ($p > 0.05$). However, although monotherapy with XRT demonstrated

significant antitumor effects in irradiated xenografts, proliferative abscopal effects were observed in lead-shielded contralateral tumors. Specifically, XRT significantly promoted growth by 23% (compared to untreated tumors) in these shielded tumors, outside the irradiated field ($p < 0.001$). Surprisingly, when CAPE was included with distant, contralateral XRT (which was not directly administered to these tumors but was administered to the contralateral flanks only), lead-shielded tumors also demonstrated a significant synergistic inhibition of growth as evaluated by early growth curve analysis ($p < 0$ .001). As with irradiated xenografts, the addition of celecoxib further inhibited tumor growth ($p <$ 0.001) making XRT, CAPE, and celecoxib the most effective regimen evaluated. Furthermore, the tumor growth inhibition was sustained, preventing tumor growth to day 50 despite these tumors not having received direct irradiation.

Proliferation Index of BxPC-3 Xenografts as Measured by Ki-67 Expression

The effect of treatment on proliferation was evaluated by using IHC to determine Ki-67 expression in xenografts harvested on Day 50. The proliferation index is expressed as a percentage of positive nuclear staining (with Ki-67) in tumor cells. As shown in Figure 5 (A and D), untreated xenografts demonstrated approximately 50% and 40% positive staining for Ki-67. In irradiated xenografts (Figure 5B), there was no significant change in Ki-67 compared to untreated groups (Figure 5A and D), with approximately 50% of the tumor cells staining positive. However, in lead-shielded xenografts in the same animals, where an abscopal proliferative effect was observed in vivo (see Figure 4B), approximately 70% of the tumor cells expressed Ki-67 (Figure 5E). For both irradiated (Figure 5C) and lead shielded xenografts (Figure 5F) xenografts, trimodal combination

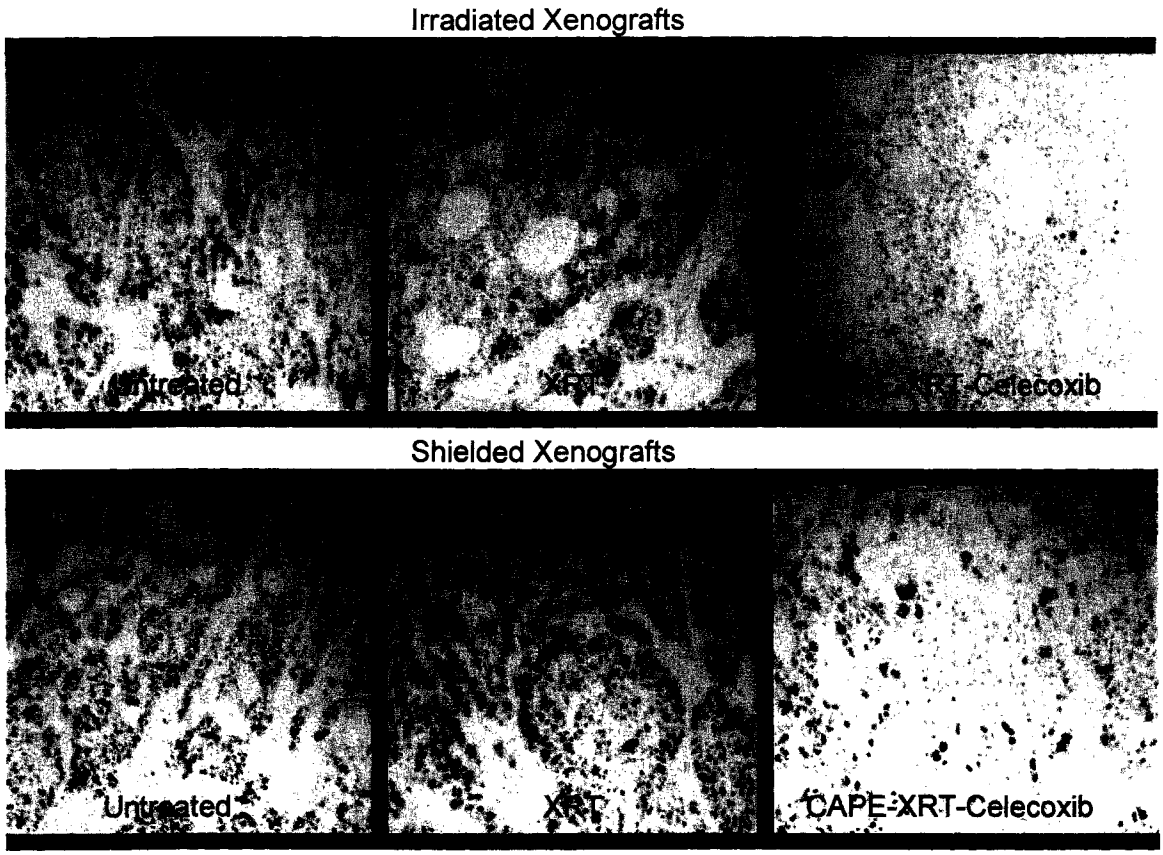

Figure 5. Immunohistochemistry of Ki-67 expression at Day 50 in irradiated (A-C) and shielded (D-F) BxPC-3 xenografts. As shown, untreated tumors (A and D) demonstrated Ki-67 staining in approximately 50% and 40% of the tumor cells, respectively. In irradiated tumors (B), 50% of the cells expressed Ki-67, similar to untreated controls. In tumors receiving the trimodal combination (capecitabine [CAPE], external beam radiotherapy [XRT], and celecoxib), proliferation was dramatically reduced (C); only 10% of the cells stained positive for Ki-67. In shielded, contralateral tumors, 70% of the cells stained positive for Ki-67 proliferation marker. (E) In shielded tumors receiving contralateral XRT-CAPE and celecoxib (F), only 20% of the cells demonstrated Ki-67 expression despite these tumors being outside the irradiation field.

(CAPE-XRT-celecoxib) caused an appreciable reduction in Ki-67 expression, with only 10 and 20%, respectively, of the tumor cells demonstrating positive Ki-67 staining.

Quantitation of TP, DPD, and COX-2 mRNA in BxPC-3 Xenografts

TP and DPD mRNA levels did not change significantly in any of the treatment groups, including groups treated with XRT ($p > 0.05$), for either irradiated or lead-shielded tumors (data not shown). COX-2 mRNA was also not significantly affected by XRT or CAPE ($p > 0.05$); however, celecoxib administration together with XRT and CAPE demonstrated a trend in decreased COX-2 expression of 2 fold, which was not statistically significant (data not shown; $p > 0.05$).

Discussion

Pancreatic cancer remains one of the most lethal gastrointestinal tumors, with an average survival of only 4-6 months and an overall 5-year survival rate of less than 10% (Brasiuniene et al., 2003; Landis et al., 1999). Despite improved endoscopic diagnostic methods (Jhala et al., 2004) and aggressive treatment regimens, only small incremental improvements in overall survival have been achieved (Brasiuniene et al.). This failure to develop an effective treatment for PAC, combined with recent advances in our ability to perform molecular analysis in biopsy-sized tissue samples, has provided the impetus to design novel treatment regimens based on the molecular profile of the tumor.

Previous studies in human colon and breast cancer xenograft models have suggested that expression of TP and DPD can be used to assess response to CAPE (a recently introduced orally administered fluoropyrimidine prodrug that mimics continuous infusional 5-FU; Pentheroudakis & Twelves, 2002). Increased TP (the final and rate-limiting

metabolic step in the conversion of CAPE into 5-FU) has been shown to result in higher intratumoral levels of 5-FU (Endo et al., 1999; Schuller et al., 2000). Preclinical studies have also demonstrated synergistic antitumor efficacy with concomitant administration of CAPE and XRT (Sawada et al., 1999). The molecular basis for synergy has been attributed to an induction of TP after XRT. Previous studies by members of our laboratory demonstrated increased TP expression in both irradiated and distant, contralateral lead-shielded xenografts (Blanquicett, Gillespie, et al., 2002). Collectively, these data offer the exciting possibility that metastatic or micrometastatic tumors outside the field of irradiation could become more sensitive to CAPE (via abscopal effects). In the current study, the antitumor efficacy of XRT alone and in combination with CAPE and/or celecoxib was examined in both irradiated and lead-shielded, contralateral BxPC-3 pancreatic cancer xenografts.

Initial studies examining PAC biopsies demonstrated statistically significant overexpression of TP and COX-2 in tumor compared to uninvolved pancreas (Figure 1; Blanquicett et al., 2003). There were no significant differences in DPD expression (Figure 1). Concordance between elevated TP mRNA and protein levels was confirmed by IHC, which demonstrated that TP protein was localized to the ductal tumor cells in PAC (Figure 2). This finding is in contrast to the CRC finding, where TP is mainly localized to the stroma (Figure 2D). On the basis of previous pharmacokinetic studies (Ishikawa, Sekiguchi, Fukase, Sawada, & Ishitsuka, 1998; Sawada et al., 1999; Schuller et al., 2000), this distribution of TP and DPD in PAC should result in selective intratumoral activation of CAPE into 5-FU (elevated TP), whereas 5-FU clearance from tumor and normal tissues should be similar (equivalent DPD levels). Collectively, these data provided the rationale for examining a multimodality treatment regimen in a preclinical animal model using

CAPE, XRT, and celecoxib. Results obtained from this study were used in the design of an ongoing Phase I clinical trial involving concurrent administration of CAPE with XRT in patients with locally advanced PAC (Saif et al., 2004).

The xenograft model used in this study (with each animal containing contralateral tumors) was designed to represent the treatment of metastatic and/or micrometastatic disease in humans, in which the "primary" tumor is irradiated and the "secondary" tumor remains outside the field of XRT (Figure 3; Blanquicett, Gillespie, et al., 2002). In irradiated xenografts, the results of monotherapy suggested that XRT was the most effective treatment, followed by celecoxib and then CAPE. However, only XRT achieved statistically significant differences from untreated controls (Figure 4). The combination of CAPE and XRT demonstrated synergistic antitumor efficacy, which was further improved with the addition of celecoxib (Figure 4). This trimodal combination demonstrated the greatest antitumor efficacy by preventing tumor growth throughout the duration of the study and, in fact, maximally decreasing tumor size by 27% (Figure 4A). The monomodal, bimodal, and trimodal therapy combinations used in this study demonstrated low toxicity with no animal deaths or significant changes in body weight (>10% from baseline).

Interestingly, abscopal effects were observed in lead-shielded contralateral tumors in the same animals. Monotherapy with XRT demonstrated proliferative effects (increasing tumor size by 23%) in lead shielded tumors outside the field of irradiation (Figure 4B). Although XRT is not typically administered as monotherapy, these results may explain the findings of a previous clinical study, in which the median survival time for PAC patients with distant metastasis treated with intraoperative radiotherapy was shorter than that of the control group (Shibamoto, Sasai, & Manabe, 1992). Of particular interest is

our finding that, when CAPE is introduced, contralateral XRT demonstrates synergistic antitumor efficacy despite these xenografts' being outside the irradiated field. Furthermore, the combination of CAPE-contralateral XRT was nearly as effective as the CAPE-XRT combination evaluated in irradiated xenografts (Figure 4A) and was also improved with the addition of celecoxib (Figure 4B). IHC analyses of Ki-67 expression demonstrated concordance with tumor area results, where significantly higher expression was observed in contralateral shielded tumors, compared to untreated BxPC-3 xenografts (Figure 5). Irradiated and contralateral shielded tumors receiving the trimodal combination demonstrated the lowest Ki-67 expression (Figure 5). These results may have significant clinical implications in the rational design of treatment regimens for pancreatic cancer where XRT is used in patients with metastatic tumors outside the field of irradiation.

Surprisingly, the synergism observed with CAPE and concomitant XRT could not be attributed to elevated TP levels in either irradiated or contralateral, shielded xenografts. A recent study of TP levels before and after XRT in endoscopic biopsies obtained from patients with locally advanced PAC also showed no significant induction of TP secondary to XRT (Saif et al., 2004). Similar results in cervical squamous cell carcinoma were recently reported (Oguri, Maeda, Yamamoto, Kusume, & Fukaya, 2004). Collectively, these data suggest that the increased TP expression observed after XRT in some tumor types (breast, colorectal and glioma; Blanquicett, Gillespie, et al., 2002; Sawada et al., 1999) may be cell specific. Furthermore, additional genes that have been associated with response to fluoropyrimidine therapy (including orotate phosphoribosyl transferase and thymidylate synthase; Isshi et al., 2002; Kubota, Watanabe, Otani, Kitajima, & Fukushiuma, 2002; Leichman, 1998; Metzger et al., 1998; Salonga et al., 2000) may need to

be examined to clarify the synergistic antitumor efficacy of CAPE and XRT. Recent advances in the ability to quantify gene expression levels (real-time low-density-array analysis; Steg et al., 2005) will allow the simultaneous examination of all known anabolic and catabolic enzymes involved in the metabolism of fluoropyrimidines and may clarify the molecular basis for response.

Elucidating the molecular basis responsible for abscopal effects has been complicated by conflicting reports in the literature suggesting that XRT can have either proliferative or antiproliferative effects in tumor cells outside the field of XRT. Proliferative effects have been attributed to an induction of matrix metalloproteinases or growth factors or to an up-regulation of the c-Met pathway in pancreatic cancer cells, which promote the malignant and proliferative phenotype of pancreatic cancer (Qian et al., 2002; Qian et al., 2003). It has been suggested that antiproliferative effects occur as a result of a cytokine-mediated antitumor effect of local XRT (Ohba et al., 1998). Unfortunately, the few clinical reports describing abscopal effects in patients are primarily descriptive (Antoniades, Brady, & Lightfoot, 1977; Nobler, 1969; Ohba et al.). In the current study, XRT demonstrated proliferative effects on distant xenografts outside the field of irradiation; because antimetabolites have been shown to be more toxic in actively dividing cells, proliferative effects of abscopal XRT may be the basis for the observed increase in sensitivity after administration of CAPE.

The results of the current study suggest a potentially efficacious trimodality regimen for the treatment of PAC; in the study, the synergistic antitumor efficacy demonstrated in irradiated xenografts with coadministration of CAPE and XRT was further improved after the addition of celecoxib. Interestingly, in xenografts outside the field of irradiation, abscopal effects were observed: (a) Increased proliferation was shown in the

absence of CAPE, and (b) Synergistic antitumor efficacy occurred after CAPE administration (and was also improved with celecoxib). These findings suggest that celecoxib may improve outcome in ongoing clinical trials of CAPE with concurrent XRT (Saif et al., 2004). Molecular analysis suggests that efficacy cannot be predicted solely from previously identified indicators of response, including TP and DPD.

These results have implications in the rational design of treatment paradigms for PAC. In this cancer, abscopal effects remain largely unknown, and metastatic disease is a primary cause of patient morbidity.

Acknowledgments

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A PHASE I STUDY OF CAPECITABINE WITH CONCOMITANT RADIOTHERAPY FOR PATIENTS WITH LOCALLY ADVANCED, UNRESECTABLE PANCREATIC CANCER: EXPRESSION ANALYSIS OF ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION BIOPSIES FOR GENES RELATED TO CAPECITABINE RESPONSE

by

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Abstract

The purposes of this study were to establish the feasibility of concurrent external beam radiotherapy (XRT) and capecitabine (CAPE) therapy and to define the maximum tolerated dose in patients with locally advanced, unresectable pancreatic cancer. Fifteen patients (median age = 66 years; male, $n = 6$; female, $n = 9$) with locally advanced panfcreatic cancer received a total irradiation dose of 50.4 Gy (1.8 Gy/day for approximately 6 weeks) and received CAPE at doses escalating (600 to 1,250 mg/m^2) twice-a-day Monday through Friday for the duration of radiotherapy. The maximum-tolerated dose was considered to have been reached when 2 or more patients in a cohort of 3 or 6 patients experienced dose-limiting toxicities. Stable and responding patients were treated with CAPE (2,000 mg/m^2 by mouth) twice a day for 14 days every 21 days (1 cycle) until evidence of progressive disease or unacceptable toxicity developed or until the patient requested that this therapy be discontinued. Restaging was performed every 3 cycles (every 9 weeks). After finishing chemoradiation, patients were assessed for tumor response and/or resectability. Tumor specimens were procured with endoscopic untrasound-guided fine needle aspiration 1 week before and 2 weeks after chemoradiotherapy to evaluate dihydropyrimidine dehydrogenase, up-regulation of thymidine phosphorylase (TP), and tumor necrosis factor (TNF)-α.

Dose-limiting Grade 3 diarrhea was observed in 2 of 6 patients treated at a CAPE dose of 1,000 mg/m^2 twice a day with concurrent external beam radiotherapy. No hematological toxicities except mild anemia were observed. The most common adverse events were Grades 1 and 2 nausea, vomiting, diarrhea, hand-foot syndrome, and fatigue. Among 12 evaluable patients, 3 achieved partial response, and 2 achieved stable disease; the overall response rate was 25%. TP was elevated during Week 2 when compared to

preradiation TP ($M = 5.8$, $Mdn = 8.1$; $p = 0.4258$). No increase in TNF-α or correlation between TP and TNF-alpha was noticed. No association between TP/dihydropyrimidine dehydrogenase ratio and efficacy of CAPE was identified as previously reported.

The recommended dose for Phase II evaluation is 800 mg/m^2 of CAPE twice a day Monday through Friday, with a concomitant radiotherapy period of about 6 weeks. This combined-modality approach proved to be a feasible and well-tolerated treatment option with promising preliminary efficacy results in pancreatic cancer. The role of TP and the TP/dihydropyrimidine dehydrogenase ratio warrants further investigation.

Introduction

Pancreatic cancer, one of the most common gastrointestinal tumors, has a 5-year survival rate of <5% (Landis, Murray, Bolden, & Wingo, 1998). Despite representing only 2-3% of the total cancer incidence, this cancer is the fourth leading cause of cancer-related death in the United States (Czito, Willett, & Clark, 2000; Lillemoe, 1995). This dismal survival rate is attributed to advanced stage at the time of diagnosis. Standard treatment for locally advanced disease is 5-fluorouracil (5-FU; either bolus or continuous infusion) and external beam radiotherapy (XRT; 50-60 Gy) as determined by GITSG and Eastern Cooperative Oncology Group (Hoffman et al., 1998; Moertel et al., 1981). The schedule which involves continuous infusion of 5-FU, requires indwelling catheters and ambulatory pumps and is more difficult to administer on an outpatient basis. Although the combined therapy increases local control and median survival from 8 to 12 months (Hoffman et al; Moertel et al.), almost all patients succumb to the disease secondary to recurrence, either local or distant. Another method uses gemcitabine as a radiosensitizer, but the best dose of gemcitabine with XRT is still not defined; neither is it known

whether this method would be superior to 5-FU chemoradiation (Crane et al., 2001). A new and promising chemoradiation approach is to combine capecitabine (CAPE) with radiotherapy.

CAPE (Xeloda, Hoffmann-La Roche, Inc., Nutley, NJ) is an oral fluoropyrimidine that generates 5-FU preferentially at the tumor site by exploiting the level of activity of the enzyme thymidine phosphorylase (TP), which is higher in tumor tissue compared with normal tissue (Miwa et al., 1998). The tumor-selective activation of CAPE potentially enhances safety by minimizing systemic exposure to 5-FU. In clinical trials, CAPE monotherapy has demonstrated a favorable safety profile that is typical of infused fluoropyrimidines (Miwa et al.; Schuller et al., 2000). The majority of adverse events were mild to moderate in severity, with a low incidence of alopecia and myelosuppression. As an oral agent, CAPE can be administered in the outpatient setting and, as a result of its rapid and almost complete absorption in the upper gastrointestinal tract, can potentially provide fluorouracil exposure similar to that of a low-dose continuous infusion of 5-FU (Schuller et al.). This process offers a more convenient therapy for patients and avoids the complications and pain associated with intravenous therapies. Two different administration schedules were explored in Phase I studies (Mackean et al., 1998; Van Cutsem et al., 2001): the intermittent schedule (2 weeks of treatment followed by 1 week of rest) with a dosage of 1,250 mg/m^2 twice-a-day (BID), which was used for further (Phase III) investigation of the use of CAPE in colorectal and breast cancer, and a 21-day continuous regimen. The results of the latter study indicated a maximum tolerated dose (MTD) of 829 mg/m^2 of CAPE BID, with hand-foot syndrome (HFS), nausea, vomiting, vertigo, abdominal pain, diarrhea, and thrombocytopenia reported as dose-limiting toxicities (DLTs; Hoff et al., 2001).

CAPE is active in pancreatic cancer as a single agent. Cartwright et al. (2002) performed a Phase II study to evaluate the efficacy of CAPE (1,250 mg/m^2/day BID for 14 days every 3 weeks) in patients with advanced pancreatic cancer. Among 38 evaluable patients, 3 were partial responses, and 11 patients had stable disease for more than four treatment cycles. Median time to progression was 111 days, and median survival was 214 days. The clinical benefit response was positive in 17% and stable in 21% of patients. Positive responses in the following areas were documented: reduced pain intensity (24% of patients), reduced analgesic consumption (12% of patients), and reduced KPS (2% of patients). Seven of 42 patients had a decrease of $\geq$20 points from baseline in their KPS score, with median time to deterioration of 57 days. Weight was maintained within 10% of baseline. The most common Grade 3/4-related adverse events were HFS (17%), diarrhea (17%), and nausea (10%; Cartwright et al.).

As an oral agent that mimics continuous-infusion 5-FU, CAPE has the potential to replace intravenous 5-FU and simplify chemoradiation for pancreatic cancer. Preclinical studies using human colon, breast, and glioma tumor xenograft models show that XRT selectively increases the activity of TP in tumor tissue but not in surrounding tissue (Blanquicett, Gillespie, et al., 2002; Sawada, Ishikawa, Sekiguchi, Tanaka, & Ishitsuka, 1999). Furthermore, the combination of CAPE and XRT demonstrated highly enhanced antitumor activity compared with either therapy alone, whereas 5-FU/XRT combination treatment showed no clear additive effect (Sawada et al.).

We report the results of a Phase I dose-escalation study conducted to determine the MTD of oral CAPE administered concomitantly with standard XRT in patients with locally advanced, unresectable pancreatic cancer. To exploit any potential up-regulation of TP secondary to XRT, tumor biopsies before and after XRT were performed.

Patients and Methods

The study was conducted according to the principles of the Declaration of Helsinki as amended in Somerset West in 1996 and according to good-clinical practice guidelines. Approval was gained from the Institutional Review Board for Human Use, and each patient gave written informed consent before being recruited into the trial.

Eligibility Criteria

Male or female patients ≥19 years of age with histologically confirmed pancreatic adenocarcinoma determined unresectable by surgeons on the basis of vascular involvement per institutional standard were eligible for this study. Further inclusion criteria were Eastern Cooperative Oncology Group performance status 0-2, adequate bone marrow function (defined as ANC $\geq$ 1.5 x $10^3/mm^3$, platelet count $\geq$ 100 x $10^3/mm^3$, and hemoglobin $\geq$ 9.0 g/dl), adequate renal function (defined as serum creatinine < 1.6 mg/dl), and adequate hepatic function (defined as a serum bilirubin $\leq$ 2 mg/dl and aspartate transaminase (aspartate amino transferase [AST], serum glutamic-oxaloacetic transaminase [SGOT]) $\leq$ 2.5 x UNL; in addition, patients were required to be capable of providing written consent.

Exclusion criteria included the presence of distant metastases such as in the liver and lungs, previous chemotherapy, or XRT or chemoradiotherapy for pancreatic cancer. The following patients were also excluded: pregnant or lactating patients; women with childbearing potential who lacked a reliable contraceptive method; patients with organ allografts; patients with significant cardiac disease; and patients with central nervous system metastases or a history of uncontrolled seizures, central nervous system disorders, or psychological disability thought to be clinically significant and to preclude informed con-

sent or adversely affect patient compliance. Patients were also excluded if they had serious, uncontrolled infections; had malabsorption syndrome; lacked sufficient physical integrity of the upper gastrointestinal tract to ensure rapid and reproducible absorption of the drug; or had known sensitivity to fluoropyrimidines.

Study Design and Treatment

The primary objective of the study was to determine the MTD of oral CAPE administered continuously BID in combination with standard XRT in patients with locally advanced pancreatic cancer; a standard escalation design was used. Secondary objectives included the evaluation of the up-regulation of TP and tumor necrosis factor (TNF)-α, the determination of the safety profile, the preliminary assessment of the antitumor activity of the combined-modality treatment, and association of the TP/dihydropyrimidine dehydrogenase (DPD) ratio with outcome.

Study treatment was started within 14 days after screening assessment. The study consisted of two parts, A and B. During Part A, patients received concurrent XRT with escalating doses of CAPE Monday through Friday over a period of approximately 6 weeks until they reached the MTD. After finishing Part A, patients were assessed for tumor response and/or resectability to potentially undergo a surgical resection. During Part B, stable and responding patients were treated with CAPE 2,000 mg/m^2 by mouth BID for 14 days every 21 days (1 cycle) until evidence of progressive disease or of unacceptable toxicity developed or until the patient requested that this therapy be discontinued. Restaging was performed every 3 cycles (every 9 weeks). Dose escalation in cohorts of 3 new patients per dose level was accomplished using a standard Phase I study design. Expansion of up to 6 patients was observed in any cohort at a given dose level or if a maxi-

mal dose of 2,500 mg/m^2/day was reached. Tumor specimens were obtained by a single experienced endosonographer with endoscopic ultrasound-guided fine-needle aspiration biopsies performed 1 week before and 2 weeks after chemoradiotherapy as previously described. Complications were assessed immediately by the endosonographer and at 2 weeks per the University of Alabama at Birmingham protocol.

Patients were irradiated once daily 5 days per week at 1.8 Gy per fraction over the course of 6 weeks. Computed tomography image-based three-dimensional treatment planning was utilized to optimize XRT treatment planning by facilitating identification of the target volume and surrounding normal structures (Figure 1). Attempts were made to minimize the XRT dose to surrounding normal tissues while ensuring an adequate dose to the target volume. Computed tomography simulation was performed with intravenous and oral contrast material to assist in localizing kidneys, liver, stomach, and intestines. Anatomical structures were contoured for dose-volume histogram analysis. The intestines were defined as the contents within the peritoneal cavity, excluding the stomach, spleen, liver, kidneys, aorta, and gross target volume, to allow for organ motion. The maximum extent of the tumor and the involved nodal areas, or tumor bed (marked with clips placed at the time of surgery), plus the adjacent locoregional nodes (celiac, peripancreatic, and portal), and para-aortic nodal areas at risk for residual microscopic diseases (clinical target volume [CTV]) were also defined by computed tomography.

XRT began on the first day of Week 1 of the study. The initial target volume received 1.8 Gy/day delivered Monday through Friday for 25 fractions (45 Gy). Typically, the edges of the initial fields were defined superiorly 1.5 cm above the CTV, inferiorly to cover the para-aortic nodes to the L3-L4 intervertebral space, laterally and anteriorly with a 1.5-cm margin around the CTV, and posteriorly by splitting the anterior vertebral

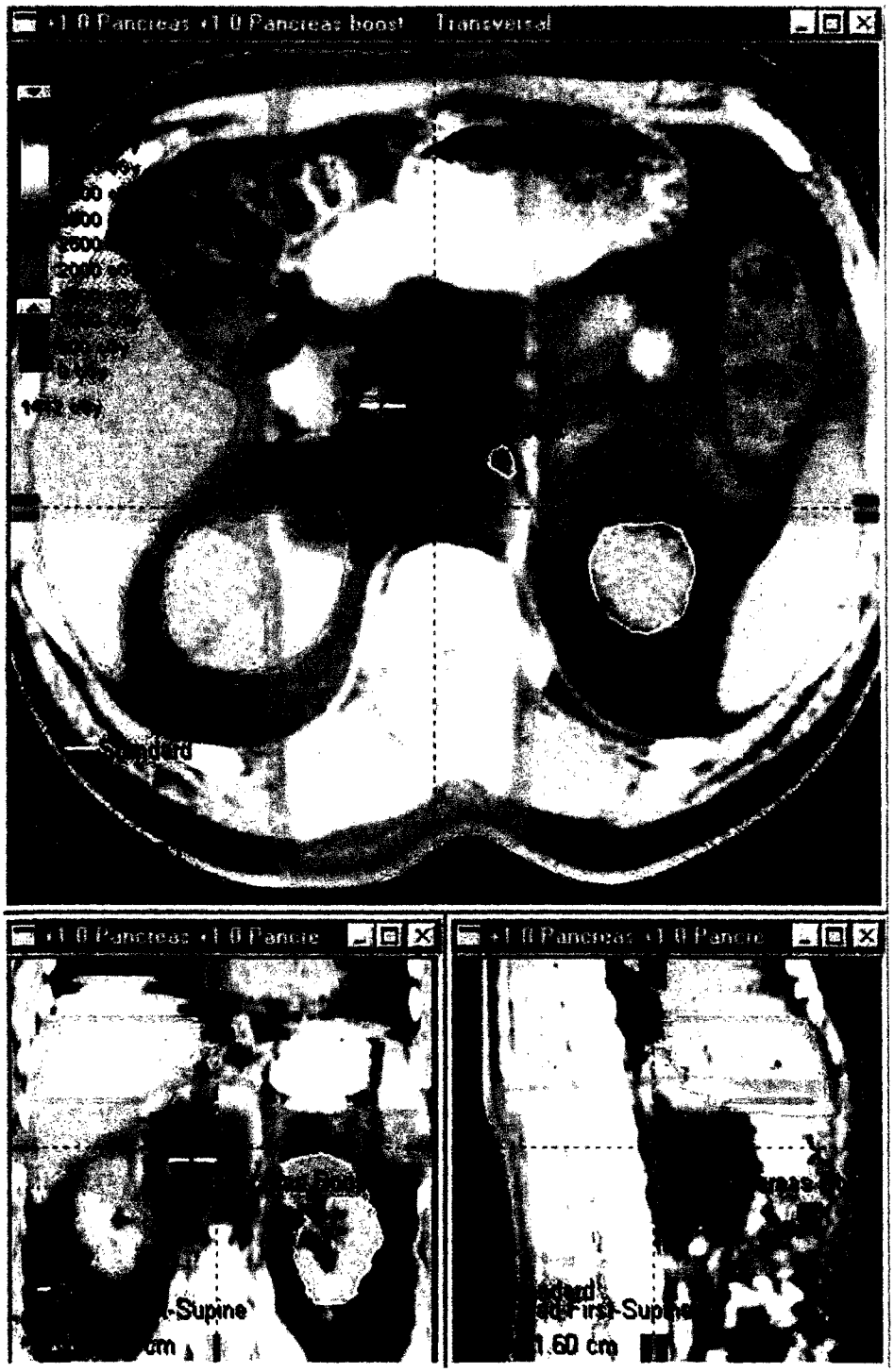

Figure 1. Three-dimensional treatment plan with color wash dose distributions identifying dose of tumor delivered to tumor volume and surrounding normal anatomical structures.

bodies in half. After 45 Gy, an additional three to five 1.8-Gy fractions were delivered to the GTV or tumor bed with a 1.5-cm margin for a total dose of 50.4-54 Gy. XRT was delivered from high-energy linear accelerators with 15-MV photon beams. Patients received 45-54 Gy (median dose 50.4 Gy) to the tumor bed.

Dose Modifications

CAPE was to be administered at escalating doses of 600, 800, 1,000, and 1,250 mg/m^2 BID Monday through Friday for the duration of XRT. The first daily dose was administered approximately 2 hr before XRT, with the second dose given 12 hr after the first. The following recommendations for dose reductions were applied: If a patient experienced a Grade 2 or 3 toxicity that was considered possibly related to CAPE treatment or clearly not related solely to XRT, CAPE treatment was interrupted, and appropriate prophylactic treatment was administered. When the toxicity resolved to Grade 0 to 1, treatment was resumed without dose adjustment. However, in the case of Grade 3 nausea/vomiting or diarrhea not resolving to Grade 0 to 1 within 2 days of interruption despite symptomatic treatment, CAPE treatment was resumed at the preceding dose level without prophylactic treatment. On the recurrence of toxicity at Grade 2 or more severe intensity, treatment was interrupted until the toxicity had resolved to Grade 0 to 1. Treatment was resumed at the preceding CAPE dose level (or at the same dose level in patients treated at the lowest dose level).

The radiotherapy schedule was not modified unless the severity of the toxicity worsened or unless a new toxicity of Grade 2 or more severe intensity occurred. If a toxicity considered to be clearly related to XRT, such as local skin toxicity, occurred at Grade 2 or more severe intensity, XRT was interrupted until the toxicity had resolved to

Grade 0 to 1 and then was resumed with appropriate prophylactic treatment if required. If the toxicity recurred at Grade 2 or more severe intensity, XRT was interrupted until the toxicity had resolved to Grade 0 to 1. The administration of CAPE was not modified unless the severity of the toxicity worsened or unless a new Grade 2 or more severe toxicity developed. If Grade 4 toxicities developed, the combination treatment was discontinued unless the investigator considered it to be in the best interest of the patient to continue treatment with XRT alone or in combination but with a reduced dose of CAPE.

Evaluation of Safety and Efficacy

Adverse events were graded according to the National Cancer Institute's Common Toxicity Criteria (Version 2.0), with the exception of HFS, which was Graded 1 to 3 (Cancer Therapy Evaluation Program, 1998). DLT was defined as the occurrence of one or more of the following conditions: any nonhematologic Grade 3 toxicity except alopecia; Grade 4 vomiting or Grade 3 stomatitis or diarrhea that does not resolve to Grade 0 to 2 within 2 days or Grade 3 HFS that does not resolve to Grade 0 to 2 within 1 week of starting symptomatic/prophylactic treatment; and Grade 4 neutropenia, Grade 3/4 neutropenic fever, Grade 3 thrombocytopenia, Grade 3 hemorrhage, or Grade 3 infection, Grade 4 hyperbilirubinemia, or Grade 3 shift in liver transaminase concentrations. The occurence of adverse events requiring interruption of CAPE for more than 2 weeks was also classified as dose limiting.

Three patients were recruited for each dose level. The CAPE dose was escalated when all 3 patients had completed a minimum of 6 weeks' treatment without DLTs, with at least one patient completing the entire treatment course. If one patient experienced a DLT, an additional 3 patients were recruited to the same dose level. Six patients were re-

cruited for Dose Level 3 (1,000 mg/m^2 BID) because of Grade 3 diarrhea that occurred in one of the first 3 patients. The MTD was defined as the dose below the dose that caused DLTs in at least 2 patients in a cohort of 6 patients.

The safety analysis included all patients who received at least one dose of CAPE in combination with one dose of XRT. All adverse events were monitored continuously during treatment and for 6 weeks after the end of treatment. Hematology and clinical chemistry were performed weekly during chemoradiation and thereafter every 3 weeks on CAPE monotherapy. After chemoradiation, tumors were evaluated on the basis of Response Evaluation Criteria in Solid Tumors (Therasse et al., 2000) criteria at baseline and 3-4 weeks after chemoradiation.

Tissue Collection and Preparation for Determination of TP, TNF-α, and DPD

Specimens of primary pancreatic ductal adenocarcinoma were obtained via EUS-guided biopsy before starting radiation on Day 1 and during Week 2 after chemoradiation. Tissues to be utilized for RNA extraction were snap frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated using the Qiagen RNA purification kit following manufacturer's instruction (Qiagen, Valencia, CA). All sample concentrations were calculated spectrophotometrically at A_{260} and diluted to a final concentration of 20 ng/μl in RNase-free water containing 12.5 ng/μl of total yeast RNA (Ambion, Austin, TX) as a carrier.

Quantitative Real-Time Polymerase Chain Reaction

Expression levels were determined using an ABI 7900 Sequence Detection System as previously described by members of our laboratory (Blanquicett, Johnson, Heslin,

& Diasio, 2002; Johnson, Wang, Smith, Heslin, & Diasio, 2000). The real-time quantitative polymerase chain reaction primers were as follows: human TP forward (5'-TCC TGC GGA CGG AAT CC-3'), reverse (5'-TGA GAA TGG AGG CTG TGA TGA G-3') and fluorophore-labeled probe (FAM-CAG CCA GAG ATG TGA CAG CCA CCG T –TAMRA); COX-2 forward (5'-GAA TCA TTC ACC AGG CAA ATT G-3'), reverse (5'-TCT GTA CTG CGG GTG GAA CA-3'), and probe (FAM-TGG CAG GGT TGC TGG TGG TAG GA-TAMRA). The sequence for the primers and probes for human DPD and S9 ribosomal have been previously described (Blanquicett, Gillepsie, et al., 2002; Johnson et al.). Expression levels were calculated using the relative standard curve method (Blanquicett, Johnson, et al.; Therasse et al., 2000). All reactions were run in triplicate, and standard curves with correlation coefficients falling below 0.98 were repeated. Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template-control reactions were performed.

Statistical Aspects

An escalation design with 3-6 patients was chosen on empiric grounds according to current standards for Phase I cancer trials (Piantadosi, 1997). The chance of not detecting a toxicity that occurs in fact in every second patient is only 1.6% in a cohort of 6 patients and less than 0.1% in a cohort of 12. According to the exploratory nature of this pilot trial, only descriptive statistical methods were used, giving rates, means with standard deviations, and quartiles and ranges.

Results

A total of 15 patients were recruited for the study at the University of Alabama at Birmingham from April 2003 to March 2004. The demographic characteristics of the patients enrolled are listed in Table 1. The patients had newly diagnosed adenocarcinoma of the pancreas found unresectable on the basis of EUS and computed tomography scan findings per institutional guidelines. There were no major deviations from the protocol; however, one patient (Dose Level 2) withdrew consent during therapy. Two other patients were taken off the study for reasons unrelated to the study: one patient developed duodenal obstruction; the second developed jaundice, the workup of which showed new liver metastases.

Table 1

Patient Characteristics

Characteristics	No. of patients ($N = 15$)
Evaluable	12
Nonevaluable	3
Gender	
Male	6
Female	9
ECOG performance status (baseline)	
0	0
1	12
2	3
Race	
Caucasian	12
African American	3
Stage	
I	0
II	1
III	14

Note. ECOG = Eastern Cooperative Oncology Group.

Dose Escalation and DLTs

Three patients were treated at each of the lowest dose levels of 600 and 800 mg/m^2 BID with no DLTs observed in any of the 6 patients. Two of the 6 patients treated with CAPE (1,000 mg/m^2 BID) experienced Grade 3 diarrhea, which occurred in one of the patients during Week 4 and in the other patient during Week 5 (Table 2).

Table 2

Patients Enrolled at Each Dose Level and Dose-Limiting Toxicity(DLT)

Dose level (mg/m^2)	Patients enrolled	Evaluable	No. of patients with DLTs
600	4	3	0
800	3	3	0
1,000	9	6	2

Only one patient treated with CAPE at a dose of 1,000 mg/m^2 BID experienced HFS (Grade 2) during chemoradiation. There were no CAPE dose reductions. Apart from the cases mentioned above and the two patients experiencing DLTs, no CAPE treatment interruptions were required. Therefore, on the basis of the DLTs, we defined the MTD as 800 mg/m^2 BID Monday through Friday, with concomitant XRT.

Hematologic Toxicity

No hematological toxicities except Grade 1 anemia were observed during chemo-radiation. All of these anemic patients were treated with Procrit and did not require blood transfusion. Grade 1/2 anemia was also seen during treatment with CAPE alone, a finding not different from observations made in other trials. Leukocytes showed no marked decrease, with the median values staying slightly above the lower limit of the normal range

(4.0/nl) throughout the study period. Platelet counts showed a similar decrease, again with no trend toward cumulative toxicity; counts below the normal range were rare.

Nonhematologic Toxicity

Adverse events according to maximum National Cancer Institute of Canada-Common Toxicity Criteria are presented for the whole patient group (Table 3). Figure 2 presents the frequency and National Cancer Institute of Canada-Common Toxicity Criteria severity grade of adverse events by body/organ system. Gastrointestinal toxicities occurred in approximately half of all patients and consisted primarily of Grade 1/2 nausea (Grade 1 in 7 patients and Grade 2 in 3 patients) and vomiting (Grade 1 in 3 patients and Grade 2 in 2 patients). Diarrhea occurred in 4 patients at Grade 1, in one patient at Grade 2, and in 2 patients at Grade 3. The latter two cases were observed at the highest dose

Table 3

No. of Patients With Nonhematologic Toxicity During ChemoRadiation (Grades 1-4)

Dose level	Nausea				Vomiting				Diarrhea				Mucositis				HFS				Fatigue			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
600	1	-	-	-	-	-	-	-	2	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-
800	2	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	2	1	-	-
1,000	4	3	-	-	2	2	-	-	1	1	2*	-	-	-	-	-	-	1	-	-	2	1	-	-

Note. HFS = hand-foot syndrome. "-" = no data available.

level (1,000 mg/m^2 BID); according to the study protocol, this event was considered to be a DLT. Grade 1 mucositis was noticed in one patient at 600 mg/m^2. Five patients developed Grade 1 fatigue, and 2 patients had Grade 2 fatigue. One Grade 2 hyperbilirubinemia at 600 mg/m^2 and one Grade 1 hyperbilirubinemia at 800 mg/m^2 were observed. Only

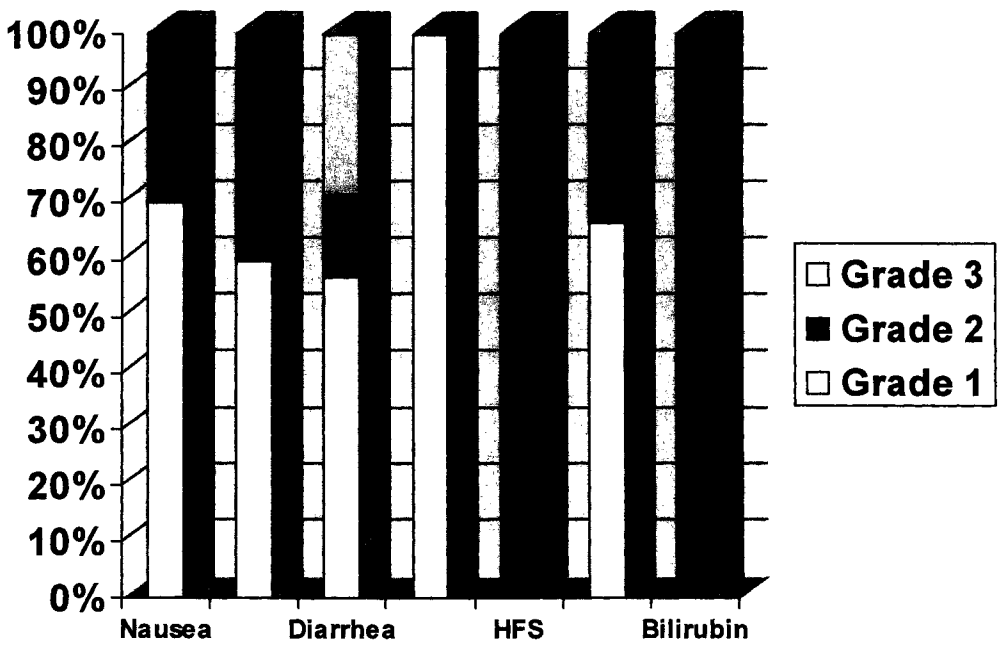

Figure 2. Frequency and Grade of nonhematological toxicities. HFS = hand-foot syndrome.

one patient developed Grade 2 HFS, which occurred during Week 6 at a dose level of 1,000 mg/m^2. No Grade 3 HFS was observed in our study as initially expected from previous studies in rectal cancer (Dunst et al., 2002; Kim, Cho, Song, & Yoon, 2002). Also, patients tolerated CAPE well when the drug was given alone; only one patient developed Grade 2 HFS, which occurred during Cycle 5, and later worsened to Grade 3 during Cycle 8. Three additional patients had Grade 1 HFS. Skin toxicity was observed in approximately 30% of patients (Grades 1 and 2), mainly in the irradiated regions, and was attributable in most cases to radiotherapy. Increased values of bilirubin without clinical relevance, a finding common in other CAPE trials, were recorded in a few patients, only one of whom was found to have metastatic liver disease. Except for the observed DLT diarrhea, no clear-cut dose/toxicity relationship was evident.

Antitumor Activity

Among 12 evaluable patients, the overall response rate was 25%; one patient achieving a partial response of 53%, another achieved a partial response of 43%, and a third achieved a partial response of 35%. No patient was found to be resectable because of persistent vascular involvement. Two patients had stable disease after finishing chemoradiation. Nine patients were continued on CAPE cycles after treatment with concurrent CAPE and irradiation. The median number of cycles administered was 6 (range = 3-17). Median survival was 14 months, with 3 patients still surviving at 20 months. One of these 3 patients is currently on second-line chemotherapy, and another is undergoing third-line chemotherapy. Most patients received gemcitabine upon evidence of progressive disease. Although there was no local disease progression, 7 patients developed progressive disease because of liver metastases.

Quantitation of TP, TNF-α, and DPD Expression

TP, TNF-α, and DPD messenger RNA was quantitated in pancreatic adenocarcinoma and uninvolved tissues by real-time quantitative polymerase chain reaction. Mean TP expression was elevated approximately 1.8-fold higher after XRT tumor samples compared to the level of expression found in before XRT tumor specimens (Table 4).

Table 4

Thymidine Phosphorylase and Tumor Necrosis Factor-α Fold Changes in Expression After External Beam Radiotherapy in Samples Collected From Pancreatic Cancer Patients

Sample	TP	TNF-α
1	0.9	0.3
2	0.8	0.4
3	-	-
4	-	-
5	-	-
6	1.2	1.2
7	-	-
8	3.7	2.2
9	-	-
10	0.2	0.5
11	1.5	0.5
12	2.0	45.8
13	1.7	0.0
14	4.7	0.2
15	1.1	0.4
M	1.8	5.1
SD	1.4	14.3
p Value (*t* test)	0.3	0.3

Note. Dashes indicate missing values, samples were not acquired either before or after external beam radiotherapy. TP = thymidine phosphorylase; TNF-α = tumor necrosis factor-alpha.

These differences were not statistically significant ($p = 0.3$). The TNF-α levels increased by 5.1 fold ($p = 0.3$). No correlation between TP and TNF-α was identified. There was no

statistically significant difference in DPD levels before and after XRT. No association between TP/DPD ratio and efficacy of CAPE was found as previously reported in studies in human cancer xenografts (Table 5).

Table 5

TP/DPD Ratio and Response in Patients Who Received Capecitabine With External Beam Radiotherapy

Patient/Dose level	Baseline TP	Baseline DPD	TP/DPD ratio	RECIST response
1/1	16.18	7.85	2.06	*SD*
2/1	108.87	306.10	0.35	PR
3/1	15.44	5.51	2.80	NE
4/1	2.31	1.03	2.20	PR
5/2	-	-	-	PD
6/2	6.01	2.10	-	NE
7/2	-	-	-	*SD*
8/2	21.83	45.05	0.40	PR
9/3	-	-	-	*SD*
10/3	141.90	26.85	5.30	DLT
11/3	15.21	6.90	2.20	PD
12/3	53.29	20.10	2.60	PD
13/3	593.99	53.58	11.00	DLT
14/3	32.11	3.05	10.50	PD
15/3	193.65	123.20	1.60	PD

Note. DPD = dihydropyrimidine dehydrogenase; TP = thymidine phosphorylase; RECIST = Response Evaluation Criteria in Solid Tumors.

Discussion

Because 2 of the 6 patients treated with CAPE (1,000 mg/m^2 BID) experienced Grade 3 diarrhea (defined as DLT), 800 mg/m^2 BID Monday through Friday with concomitant XRT is the recommended dosage for subsequent trials. Interestingly, no Grade 3 HFS as a DLT was observed in our study but was seen in similar studies conducted in rectal cancer (Dunst et al., 2002; Kim et al., 2002). Patients enrolled in our study were

administered pyridoxine at a dosage of 50 mg by mouth, three times a day (Beveridge, Kales, Binder, Miller, & Virts, 1990; Fabian et al., 1990), udder balm was applied BID (Lassere & Hoff, 2004). Either this preventive approach or a difference in the level of TP in different tumor sites may be responsible for the difference in the observation of this toxicity.

Pancreatic cancer remains one of the most fatal gastrointestinal tumors; the median average survival of persons with this disease is only 4-6 months. Treatment with XRT or chemotherapy remains limited because toxicity to the gastrointestinal tract and kidneys occurs with the former method and because no increased survival occurs with the latter method. This poor response to therapy led to recent advances in the molecular analysis of tumor tissue to quantify drug-metabolizing enzymes as a way to elucidate potential indicators of response to chemotherapy. Data from human cancer xenograft studies suggest that XRT up-regulates TP in tumor tissue (Sawada et al., 1999). A single fraction of XRT (2.5 to 5 Gy) resulted in significant increases in TP in four of five xenograft models. A 9.4-fold increase in tumor TP levels was observed after whole body irradiation, but no increase in liver TP levels was found. An increase in tumor levels of TNF-α, another known up-regulator of TP, was also observed to precede the increase in tumor TP levels, suggesting a cytokine-mediated mechanism. Sawada et al. also demonstrated the effects of combined CAPE-XRT on tumor growth using a WiDr human colon cancer model, which is known to be refractory to 5-FU because of low TP levels. The anittumor effect of CAPE-XRT appeared to be more than additive. In contrast, the antitumor of 5-FU-XRT was less than additive (Sawada et al.). No statistical elevation of TP or TNF-α was noticed in our study. Similarly, no correlation between TP and TNF-α levels was found. It may be possible that either TP up-regulates directly after XRT or TNF-α is not

the only mediator. Similar studies in BxPC-3 xenografts in our laboratory demonstrated synergistic effects of CAPE with concomitant XRT but showed no induction of TP levels in either irradiated or contralateral shielded BxPC-3 xenografts. Although these results support the concurrent use of CAPE and XRT in pancreatic cancer, there appear to be additional genes (other than TP and DPD) associated with response to CAPE alone and to CAPE with XRT. The major benefit of CAPE lies in its favorable toxicity profile; its oral administration; and, most importantly, its activity in pancreatic cancer, both and as a single agent and as a radiosensitizer. Although this first prospective study of CAPE with radiation in pancreatic cancer included a small number of patients, the results indicate that CAPE can be a safe and equally efficacious replacement for intravenous 5-FU.

The major pitfall in the use of any oral agent lies in patients controlling their medication. In this study, only one patient made mistakes in dosing; this patient was removed from the study because of safety issues. During each visit, patients were asked to sign and write in a journal the time at which they took their CAPE.

In conclusion, the use of CAPE and concurrent XRT for locally advanced pancreatic cancer appears to be safe and well tolerated and to lack unexpected toxicities. The MTD was defined as 1,600 mg/m^2/day (800 mg/m^2 BID). Oral CAPE has good bioavailability, can be administered orally, and has a tolerable side-effect profile. The rates of tumor response and survival were comparable with if not better than the rates found for standard treatment with infusion 5-FU; however, to test our findings vigorously, a Phase III trial comparing the two treatment routes is needed. Additionally, CAPE has the convenience of oral administration and avoidance of catheter-related problems. We are currently performing a Phase II study to further confirm the safety of this regimen and to de-

termine the effect of XRT on TP and the relation between TP/DPD ratio with CAPE therapy.

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SUMMARY AND CONCLUSIONS

Discussion

In most developed countries, including the United States, one in four deaths result from cancer, with a total of 1,372,910 new cancer cases per year. In the United States alone, 570,280 deaths are expected in 2005 (Jemal et al., 2005). Despite the significant advances that have been made in cancer research, the outcome of this disease remains poor. Modern, emerging techniques and novel approaches need to be applied in the cancer research field in order to further advance it. Pharmacogenomics constitutes one such novel approach. In this dissertation, we describe the successful application of pharmacogenomics, where we design a potentially synergistic treatment regimen (composed of CAPE-XRT) based on the molecular profile of GBM and PAC tumors. This treatment regimen was possible by conducting in vitro, preclinical, and clinical studies that employed carefully validated gene expression methods.

The recent mapping of the human genome, coupled with quantitative methods for determining gene expression, and the current availability of quantitative microarrays to study global gene expression, has greatly increased the potential for future progress in the understanding and the treatment of cancer at a molecular level. The introduction of RT-Q-PCR is one example of the advancement in our ability to accurately quantitate gene expression levels in small tissue samples (such as the specimens obtained with needle biopsy-sized endoscopic ultrasound-guided fine needle aspiration). Gene expression analysis via this method has demonstrated having an important role in the study of cancer,

where differences in gene expression levels have been associated with tumor metastasis (Jackson & Puisieux, 2000; Weber & Ashkar, 2000), tumor progression (Bowen et al., 2000), and systemic toxicity (Johnson et al., 1999). However, a critical requirement in the accurate quantitation of gene expression is the availability of a ubiquitously expressed HKG to normalize the amount of total RNA in each sample. Although recent pharmacogenomic studies suggest that response to chemotherapy (particularly in colon cancer patients treated with 5-FU) may be predicted by quantitation of drug-metabolizing enzymes (Diasio & Johnson, 2000; Ishikawa et al., 1999; Kirihara, Yamamoto, Toge, & Nishiyama, 1999; Lenz et al., 1998; Salonga et al., 2000), the accuracy of these studies may be jeopardized if the HKG selected demonstrates significant variability. Our first specific aim was to utilize RT-Q-PCR to evaluate the variation in expression among 15 different HKGs in normal versus carcinomatous tissues to identify an "optimal" HKG (one demonstrating <2-fold changes in expression between tissues) for use in pharmacogenomic studies involving malignant and metastatic tissues.

Comparative analysis of 15 HKGs in normal and carcinomatous tissues revealed dramatic differences not only among these tissues but across malignant tissue types (e.g., primary versus metastatic). Compared to normal colonic tissue, primary carcinomatous colon samples demonstrated <2-fold differences in expression among the 15 HKGs examined. In contrast, there was significant variation in HKG expression in metastatic versus normal liver samples. These data emphasize the need to evaluate HKG variability (in the tissue of interest) before conducting pharmacogenomic studies. For example, although glucose-6-phosphate dehydrogenase or β-actin could be used as appropriate HKGs when comparing normal and carcinomatous colon samples, their use with carci-

noma metastatic to the liver would result in a significant underestimation of total RNA, thereby affecting the accuracy of gene expression studies performed in those tissues.

Nevertheless, some generalizations could be made from our HKG study. For example, it was revealed that, as a group, HKGs that code for metabolic enzymes demonstrate the highest overexpression in carcinomatous versus normal tissue samples. These data agree with previous findings of elevated levels of the metabolic enzymes glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase or both in hypermetabolic states such as lung, pancreas, prostate, hepatic, and cervical cancers (Chang, Juan, Yin, Chi, & Tsay, 1998; Chesnokova, 1990; Thellin et al., 1999). Another generalization that could be made is that nonmetabolic (ubiquitin, GUS, β-2m, TBP, CYC, huTfR) and structural (β-tubulin, β-actin) HKGs demonstrate less variation than the metabolic HKGs do. For example, β-actin demonstrated intermediate variability relative to the metabolic enzymes in liver metastases relative to normal liver. However, because β-actin is as a commonly used HKG, it is noteworthy that it demonstrated any variability whatsoever. Nonetheless, when the increased requirements for cytoskeletal proteins in tissues where cellular proliferation or motility is occurring (i.e., cancer) are taken into consideration, β-actin's increased expression in cancerous tissue is not entirely unexpected. Our analyses agree with those of previous studies demonstrating variability in expression of β-actin in different tissues and disease states (Harrison et al., 2000; Suzuki, Higgins, & Crawford, 2000; Thellin et al., 1999; Yuan, Rosenberg, Ilieva, Agapitos, & Duguid, 1999). In our analyses, in addition to β-actin, ubiquitin, β-2m, TBP, β-tubulin, CYC, and huPO also demonstrate intermediate variation which ranges from 2 to 4.8 fold (average increases in liver metastasis compared to normal liver). In particular, ubiquitin,

which is also a commonly used HKG, demonstrates variability of expression that may result from its involvement in the proteolysis pathway in cyclin-dependent kinase activity occurring during cell division (Loda et al., 1997; Montagnoli et al., 1999; Tanaka, Suzuki, & Chiba, 1998).

In addition to the aforementioned general findings, the conclusion that ribosomal HKGs such as 18S and S9 demonstrate the least variability (less than 2 fold), irrespective of tumor or tissue type, is of great utility. This low variability may result from their lack of involvement in cellular metabolism (Finnegan, Goepel, Hancock, & Goyns, 1993; Kotter & Entian, 1995). However, exceptions to the generalizations described above were also found. For instance, the human transferrin receptor huTfR (involved in cellular metabolism) did not demonstrate significant variability in colorectal tissues yet demonstrated overexpression in liver metastasis (compared to normal liver). Transferrin receptor mRNA levels have been shown to increase during cell proliferation (Wang, Li, & Chen, 1999), and rapidly growing cells require more iron for their growth and metabolism. Interestingly, previous studies have suggested a prognostic value in the expression of tranferrin receptor in breast cancer (Yang, Wang, Elliott, & Head, 2001), as well as in hepatocellular carcinoma (Beckman, Hagerstrand, Stenling, Van Landeghem, & Beckman, 2000). However, the high variability of huTfR between tissue types in these analyses demonstrates that it is not an adequate HKG for liver metastasis but may be one for CRC, further suggesting that the function of an enzyme cannot be used as an indicator of HKG variability. This further emphasizes the need to validate a particular HKG in the tissue of interest, before undertaking gene expression analysis. Having a validated HKG should facilitate comparisons between normal and carcinomatous tissue by diminishing

potential errors introduced by variable HKG expression. The approach described herein could be used to validate other HKGs in other tissue types and/or disease states.

By following our methodology, we also determined HKG variability between normal and tumor brain samples (GBM), demonstrating previously identified trends. For example, ribosomal HKGs (18S and S9) also demonstrate <2-fold differences in expression between normal and GBM tissues, whereas metabolic enzymes (glucose-6-phosphate dehydrogenase, phosphoglycerokinase) demonstrate greater than 2-fold differences. Having a greater level of precision in our gene expression quantitation methods ascribes validity to our molecular-profiling results in GBM. Consequently, having performed careful gene expression analyses in GBM tissues provided an insight into potential biological targets and, ultimately, potential chemotherapeutic strategies for this disease.

The overexpression of TP observed in GBM was particularly noteworthy because of this enzyme's involvement in CAPE metabolism. Because CAPE is primarily approved for the treatment of colorectal and breast cancers, we also examined TP and DPD levels in CRC compared to normal colon in 100 human tissue samples, as reference of comparison (Wang, Johnson, Blanquicett, Heslin, & Diasio, 2002). Interestingly, the TP/DPD ratio is approximately 2-fold higher in CRC tissue than in normal colonic mucosa. On the basis of our analyses, this difference translates into a TP/DPD ratio that is 8-fold higher in GBM relative to CRC and to normal tissues. This finding suggests that GBM could be potentially treated with CAPE; furthermore, this profile appears more suitable than the one observed in CRC (16-fold higher TP/DPD ratio in GBM compared to 2-fold higher in CRC and relative to normal tissues), where CAPE is already approved and efficacious.

Previous data had suggested that it was possible to up-regulate TP with a concurrent increase in CAPE efficacy via several mechanisms including cytokines; chemotherapeutics; and, more recently, XRT (Eda et al., 1993; Endo et al., 1999; Sawada et al., 1998; Sawada, Ishikawa, Sekiguchi, Tanaka, & Ishitsuka, 1999). Preclinical studies have shown this effect to be synergistic, unlike the combination of 5-FU plus radiotherapy, which produces only additive efficacy (Sawada et al., 1999; Wilke, 2002). The current standard of care for the treatment of GBM remains XRT (Andratschke, Grosu, Molls, & Nieder, 2001). Although XRT has been shown to increase TP in colon, cervix, gastric, and breast cancer xenograft models (Sawada et al., 1999), the effects in glioma xenograft models had not been examined before our study. Furthermore, because intratumoral TNF-α was shown to increase before TP did, which implied that TNF-α induced TP expression (Sawada et al., 1999), we examined the effect of XRT on the expression of TP, DPD and TNF-α in both irradiated and lead-shielded U87MG glioma xenografts (using a xenograft model designed to represent distant and/or micrometastatic disease in humans) in our effort to meet Specific Aim 2. Determination of the effect of XRT on the intratumoral TP/DPD ratio in a glioma mouse model of brain metastasis could potentially provide information about the possibility of enhancing CAPE as a therapeutic option for GBM. In this study, we demonstrated that TP mRNA is indeed upregulated by XRT, both in irradiated and shielded tumors. Since relapses in persons with malignant brain tumors are commonly observed after radiotherapy (Wild-Bode, Weller, Rimner, Dichgans, & Wick, 2001), the induction of TP in shielded tumors that were not directly irradiated has potential implications for improving CAPE efficacy in patients with distant metastatic or micrometastatic tumors. This study also revealed that an increase in TP expression is not

observed in mouse liver and agrees with previous studies, which suggest that the induction of TP by XRT is possibly tumor associated (Sawada et al., 1999).

Increased TNF-α has been proposed as a potential mechanism for increased TP levels after XRT of several xenografts (Sawada et al., 1999). Interestingly, TNF-α mRNA levels did not increase with XRT. In our study, XRT of glioma xenografts increased IFN-γ (6.3 fold), IL-10 (3.7 fold), and IL-1α (1.6 fold) mRNA levels, compared to the levels found for non-irradiated control tumors. No significant increase in cytokine mRNA levels was detected in shielded tumors in the same animals.

Taken collectively, these data suggest that the molecular basis for increased TP mRNA levels may vary depending on tumor type and cytokine profile. Although TNF-α may play a greater role in TP induction in colon cancer xenografts, IFN-γ and IL-10 appear to be important cytokines in U87MG glioma xenografts. In addition to TNF-α, IFN-γ has also been previously reported to induce TP (Sawada et al., 1999). In fact, more than 200 genes and macrophages have been reported to be activated by IFN-γ (Goto et al., 2001), implicating a complex signaling system for this cytokine. Additionally, IFN-γ has been shown to have antitumor activity in recurrent gliomas by inhibition of angiogenesis, apoptosis of endothelial cells, suppression of glioma growth, and decreased cell proliferation (Knupfer, Poppenborg, Van Gool, Domula, & Wolff, 1997). Urbani, Maleci, La Sala, Lande, and Ausiello (1995) suggest that there is defective mRNA expression of IFN-γ in glioma patients; thus, an increase of IFN-γ by XRT could be advantageous to a patient because of its induction of TP (provided the patient is receiving CAPE), as well as its direct antitumor properties. Whether IFN-γ causes an up-regulation of TP, either indirectly by macrophage activation or directly by transcriptional mechanisms, would need to be clarified (as we attempt to do in our subsequent studies); however, the fact that IFN-γ is

observed to increase the most after XRT of glioma xenografts implicates a potential candidate contributing to TP up-regulation and may concord with studies that have identified IFN-γ as the cytokine that most effectively increases TP expression, relative to TNF-α and IL-1α (Goto et al., 2001).

IL-10, which is a potent anti-inflammatory cytokine, had not been previously reported to be involved in TP induction but was observed to increase after XRT in our study. Interestingly, IL-10 is an inhibitor of TNF-α production. The molecular pathways by which IL-10 inhibits TNF-α production remain unclear despite the fact that diverse mechanisms have been published (Denys et al., 2002). The increased levels of IL-10 we observed may explain the lack of increase in TNF-α mRNA levels in irradiated xenografts. As has been described above, results of other studies have suggested an impaired, cell-mediated immunity in patients with gliomas as a result of immunosuppressive factors secreted by the tumor; interestingly, IL-10 happens to be an immunosuppressive cytokine (Dix, Brooks, Roszman, & Morford, 1999). IL-1α also increased in our glioma xenografts. The increase in IL-1α after XRT observed in our study is in agreement with other findings implicating IL-1α in TP up-regulation (Sawada et al., 1999). However, we observed less than 2-fold increases of this cytokine during the first 24 hrs after XRT and no further increases afterward.

In shielded xenografts, IFN-γ mRNA levels were not detected at 4, 6, and 10 days after XRT; however, protein levels and activity may require further investigation. IL-10 and TNF-α mRNA levels were detected but were not significantly higher (<2-fold increase) in shielded tumors relative to nonirradiated/control tumors (data not shown). Nevertheless, the prolonged and sustained increase in TP mRNA levels (up to 20 and 15 days in directly irradiated and shielded tumors, respectively) has significant implications

for improving CAPE efficacy with XRT. The dramatic and prolonged up-regulation of TP is also suggestive of the involvement of molecular mechanisms such as stabilization of the TP RNA transcript (as demonstrated by our subsequent studies). Because CAPE mimics continuous infusion, the fact that such high levels of TP can be achieved with XRT (and for such a prolonged time) may be quite advantageous to a patient receiving CAPE chemoradiotherapy.

One consideration (and potential limitation) in proposing CAPE chemoradiation for GBM involves the blood-brain barrier (BBB) and central nervous system penetration. The role of the BBB in preventing adequate intratumoral drug concentrations remains a valid concern in the use of some chemotherapeutic agents, including CAPE. It is not known whether CAPE can cross the BBB (Roche Pharmaceuticals, 2003). However, what is known is that the chemical structure of CAPE indicates that the CAPE molecule and its metabolites are more lipophilic than the active 5-FU moiety is found to be. Furthermore, recent studies suggest that the BBB is substantially disrupted in disease states because the existing endothelial abnormalities in the microvasculature of brain tumors and other pathological states of the brain allow nonlipid-soluble substances and other compounds (which normally would not penetrate an intact BBB) to demonstrate activity against central nervous system metastases (Lin, Bellon, & Winer, 2004). This is evidenced by the enhancement of brain lesions with water-soluble contrast agents such as gadolinium. Also, the fact that high response rates have been achieved with agents that do not easily cross the BBB, such as the antineoplastic, cisplatin (in patients with central nervous system and germ cell tumors), or antibiotics (penicillins and third-generation cephalosporins) that penetrate an intact meninges poorly but achieve efficacy in the inflamed meninges (meningitis), illustrates the possibility of achieving response in a patho-

logical state (such as that in brain tumors) by using CAPE or chemoradiotherapy. However, the most compelling evidence supporting the possibility that CAPE may penetrate the BBB comes from two case reports. One report demonstrates efficacy of the drug in brain metastasis originating from breast carcinoma after 2 months of treatment (Wang, Yung, Royce, Schomer, & Theriault, 2001), and another recently published case report describes the "complete response of brain metastases originating in breast cancer to CAPE therapy" (Siegelmann-Danieli et al., 2003, p. 833). Additionally, preliminary results of a Phase I trial performed at our institution (involving an examination of CAPE chemoradiotherapy) also suggest that this may be a feasible treatment option for GBM (Newman et al., 2004).

In addition to the BBB, drug-metabolizing enzyme induction plays a special role in the treatment of brain disease and should be mentioned. It has been suggested that induction of enzymes involved in drug metabolism can affect the pharmacokinetics of agents employed in the treatment of high-grade gliomas (where the BBB is of concern). For example, patients on paclitaxel and 9-aminocamptothecin have demonstrated altered concentrations of these agents as a result of concomitant administration of anticonvulsants, which induce hepatic oxidation pathways (Galanis & Buckner, 2000). This finding exemplifies the importance of the induction of drug-metabolizing enzymes in chemotherapy, as we have attempted to illustrate. We specifically demonstrate substantial induction of TP after XRT, suggesting a significant potential for intratumoral drug activation in regions (e.g., the brain) that may otherwise not achieve substantial drug concentrations.

Taken collectively, the findings of our GBM study demonstrate differential expression of DPD and TP in normal and neoplastic brain tissues and suggest that differentially regulated molecular mechanisms may control the expression of important drug-

metabolizing enzymes in different tissues. The high TP/DPD ratio observed in brain tumor tissues compared to adjacent normal tissue (along with the fact that this ratio may be improved by a combinatorial approach such as including XRT) may offer an exploitable opportunity to enable tumor-specific activation of newer antimetabolites such as CAPE. This activation may have significant implications for diminished toxicity and highly specific tumor cell targeting. Our approach may also provide the basis for the development of tumor- or patient-specific treatments, as well as for the development of CAPE for use in other tumor types (i.e., those having the molecular profile warranting CAPE treatment). Additionally, enhanced efficacy may be achieved in patients treatable with CAPE, and perhaps nonresponsive patients may achieve response via XRT and subsequent increased TP expression. Even more promising is the consideration that this effect may possibly extend to distant or multiple-metastatic tumors.

Insight into the putative, tumor-associated pathways regulating TP expression may allow the improvement of CAPE efficacy and the development of novel anticancer agents targeted at the tumor. Modulation of existing therapies to specifically enhance drug activation within the tumor cells or tissues represents a significant advantage in cancer chemotherapy and could be achieved through an examination of the molecular mechanisms involved. To complete our third specific aim, we attempted an elucidation of the mechanisms responsible for the observed TP up-regulation by XRT in glioma xenografts. These studies reveal that cytokine-mediated transcriptional and posttranscriptional mechanisms may be responsible for the increase in TP expression after XRT induction of cytokines. A similar tactic can be used to determine the mechanisms regulating TP in pancreatic cancer cells (where TP is not observed to increase with XRT). In-depth studies of the regulation of TP, such as epigenetic changes affecting its expression, will also be

useful. Because our mechanistic studies could not definitively prove that cytokines, specifically tumor-secreted cytokines, are responsible for the observed up-regulation of TP in irradiated (and shielded) glioma xenografts (in vivo), the conclusions that can be made are limited. However, these mechanistic studies do suggest that cytokines such as TNF-α or IFN-γ have effects on TP mRNA in an isolated, in vitro system. Additional experiments that could have been performed to test our hypotheses involve the administration of cytokine antibodies (such as antihuman IFN-γ) to mice and the determination of the effect of this component on TP up-regulation. If blocking the effects of cytokines with antibodies abolishes TP up-regulation by XRT, it could be stated with greater confidence that cytokines are regulating TP expression in vivo. Another limitation of the studies in which we demonstrated that TP is up-regulated and attributed this up-regulation to cytokines (which are thought to be originating from the tumor), is the lack of determining the inflammatory effects of XRT alone (without tumor tissue involved) on TP. One of the main theories proposed to explain abscopal effects postulates that local XRT induces a release of cytokines into the circulation that can then mediate a systemic effect (Ohba et al., 1998). The inflammatory response after XRT can in itself result in the secretion of cytokines, suggesting the possibility that a tumor need not be present. Leukocytes of the immune system, such as monocytes/macrophages, mast cells, and dendritic cells, play important roles in acute inflammation because they release inflammatory mediators (Wedemeyer, Tsai, & Galli, 2000). Monocytes/macrophages differentiate into immature dendritic cells, which can migrate into inflamed peripheral tissues. Once activated, macrophages are a major source of growth factors and cytokines that can "profoundly" affect endothelial, epithelial, and mesenchymal cells in the local mircoenvironement

(Robinson & Coussens, 2005); therefore, tumors may not be necessary for the production of cytokines after XRT.

In angiogenic vasculature associated with tumor development, however, a common feature observed is that of enhanced expression and/or bioavailability of chemokines and cytokines in such neoplastic environments (Conti, Dube, & Rollins, 2004). It should be considered that tumors are composed not only of neoplastic cells but also activated stromal cells, including endothelial and vascular smooth muscle cells, which may be interacting with the tumor cells. Leukocytes also comprise a large percentage of the total cellular repertoire in many tumor types (Robinson & Coussens, 2005), all of which are capable of producing a myriad of cytokines and soluble mediators. The production of these cytokines in neoplastic microenvironments can significantly enhance inflammatory responses (Balkwill, 2002; Balkwill & Mantovani, 2001). This capability suggests that neoplastic tissue is a chemokine/cytokine-rich environment that can augment the effects, the production, and the bioavailability of cytokines. Nonetheless, we could have addressed (in part) the limitation of our study by irradiation of a limb lacking the presence of any tumor cells. Camphausen et al. performed this experiment in 2001, and they demonstrated that irradiation of a limb indeed has an effect on a distant tumor, further suggesting that the effect is not necessarily tumor specific. It would be informative to determine how the presence or absence of a tumor (or tumor type) could affect Camphausen et al.'s results, as well as ours. One point of interest that must be noted is that we implanted human tumor cells in mice. The cytokines (mRNA levels) detected in irradiated glioma xenograft tissue were specifically human. Furthermore, human IFN-γ is highly species specific and is biologically active only in human and primate cells (R&D Systems, Minneapolis, MN). Therefore, one can speculate whether simply irradiating a murine limb or

tissue would result in the increased production of human cytokines, suggesting the involvement of the human tumor.

Nevertheless, regardless of the precise mechanisms responsible for TP induction, the promising results obtained in our GBM metastatic model suggested that it is theoretically possible to enhance chemotherapy efficacy via drug metabolism induction (in a highly lethal tumor type resistant to therapy) and were worthy of examination in other lethal and treatment-resistant tumor types (besides GBM). Highly lethal cancers such as GBM or PAC have such few chemotherapeutic options and survival time after diagnosis is so short that potentially efficacious treatment strategies in one cancer type should be (and are commonly) examined in other cancer types. Furthermore, the hypothesis that CAPE chemoradiation could be effective in tumors expressing TP, required confirmation.

Preliminary analyses of PAC clinical samples suggested that TP was overexpressed and localized to the ductal tumor cells. Metastatic disease is the primary source of morbidity in PAC patients. Consequently, we evaluated CAPE chemoradiation in a PAC xenograft model representing metastatic disease. In that study (limited to accomplish Specific Aim 4), a CAPE-XRT treatment regimen proved to be synergistic and highly efficacious. This synergy was enhanced when celecoxib was included. As a matter of fact, the trimodal combination of CAPE-XRT-celecoxib not only prevented tumor tripling by Day 50 but decreased tumor size to a maximum 73% of its original size. This effect was sustained, and, surprisingly, was also observed in contralateral tumors that had not received XRT (as the results of our glioma studies suggested would be the case). Surprisingly, XRT did not appear to affect TP levels in these xenografts despite an observed synergistic antitumor effect of CAPE-XRT. This lack of effect was confounding and may perhaps be attributed to a greater resistance of pancreatic cancer cells to XRT

relative to glioma cells. It can also be hypothesized that other genes besides TP (and DPD) are involved in the response to CAPE or CAPE combinations. For example, the enzyme orotate phosphoribosyl transferase contributes to 5-FU phosphorylation, and orotate phosphoribosyl transferase activities within cancer cells may predict sensitivity to 5-FU (Isshi et al., 2002). Other investigators have shown that high mRNA levels of both thymidylate synthase (Leichman, 1998) and TP (Metzger et al., 1998) in pretreatment tumor biopsies could identify tumors that are nonresponsive to 5-FU-based therapy (Salonga et al., 2000). In addition, other enzymes such as DNA repair enzymes, drug-metabolizing enzymes, and or growth factors may independently or in association affect the response to CAPE and would need to be examined in future studies.

The promising results of our preclinical studies now warranted evaluation or translation into the clinic, the goal of our fifth specific aim. The evidence available to that point provided the foundation for the initiation of two clinical trials at our institution. Interestingly, after the completion of one of the trials in which we examined CAPE-XRT in GBM patients, low toxicity was reported; remarkably, one complete remission was documented. Furthermore, all patients survived for more than 1 year. As discussed in detail in this dissertation, another Phase I clinical trial to examine CAPE with concomitant XRT in patients with locally advanced, unresectable pancreatic cancer was conducted at our institution. That trial demonstrated that a CAPE chemoradiation combination is both active and tolerable, with only mild toxicities in PAC patients. Surprisingly, grade 3 hand and foot syndrome, a common side effect of CAPE, was virtually absent in that study. Results in clinical samples demonstrate the localization of TP as confined to the ductal tumor cells of PAC. This observation may possibly explain the amelioration of toxicity (such as hand and foot syndrome, which is common in CRC, where TP is mainly local-

ized to the stroma) in PAC patients. Additionally, TP and TNF-α levels were also evaluated before XRT and after XRT in the PAC clinical trial. However, neither TP nor TNF-α was shown to be significantly upregulated by XRT, corroborating our findings in PAC preclinical studies (and unlike our GBM xenograft results). These results further emphasize the need to thoroughly examine the molecular regulation of TP and suggest that this regulation may be tumor type dependent.

In contrast to the PAC clinical study, preclinical studies in xenografts included celecoxib examination, as a result of the significant over expression of COX-2 mRNA levels found in PAC relative to normal pancreas. This significant difference represents an opportunity to use inhibitors (i.e., celecoxib) to selectively target tumors highly expressing COX-2 (such as PAC). Celecoxib has been suggested to enhance the response to both chemotherapy and radiotherapy and to enhance tumor growth inhibition via suppression of COX-2, representing the potentially increased benefit of adding celecoxib to CAPE-XRT chemoradiation (Crane, Mason, Janjan, & Milas, 2003). Although celecoxib significantly improved the antitumor effects of CAPE-XRT in the PAC preclinical studies, it remains to be determined whether this improvement would be the case in a clinical setting. This examination may be complicated by recent controversy regarding the use of COX-2 inhibitors. Adverse events with the specific COX-2 inhibitor, celecoxib, are unclear relative to the clearly-demonstrated adverse events seen with rofecoxib (Graham et al., 2005; Levesque, Brophy, & Zhang, 2005). Furthermore, adverse events resulting from the use of celecoxib mainly occur in populations susceptible to cardiotoxicity or renal disorders (ischemic heart disease, hypertension, etc.) and at high doses (Savage, 2005). Recent Food and Drug Administration evaluation of celecoxib for use in arthritis patients has determined that its benefits are greater than the potential risks posed are

found to be, thus resulting in the agency's decision against removing celecoxib from the market. Logically, the lethality of PAC may have consequences greater than those of the possible adverse effects of celecoxib. Our results suggest improved efficacy of chemotherapy with celecoxib, further supporting the use of this drug for potentially improving cancer treatments (which generally involve classes of drugs that are more toxic than COX-2 inhibitors may be). Thorough evaluation of celecoxib is still needed to conclusively determine its potential adverse effects; however, to reiterate, these would appear small in comparison to the benefits of improving cancer chemotherapy, where standard treatments oftentimes fail.

In summary, our clinical studies establish a "proof of principle" by confirming earlier studies proposing the concurrent use of CAPE-XRT in GBM and pancreatic cancer patients as a rational combination. Additional efforts are required to identify other genes associated with response to CAPE, examine celecoxib inclusion in CAPE-XRT regimens, and determine the molecular mechanisms responsible for the regulation of TP in PAC or other tumor types. In conclusion, the results of the studies included in this dissertation establish and support the rationale for a CAPE chemoradiotherapy regimen to treat PAC and GBM, that is based on the molecular profiles exhibited in those tumor types. Furthermore, this oeuvre elucidates to the mechanisms involved in the regulation of the CAPE-metabolizing enzyme, TP. A greater understanding of the regulation of TP (and other enzymes associated with the response to CAPE) may allow a better tailoring and optimization of the efficacy of CAPE (whether given alone or in combination). This should ultimately improve outcome not only in malignancies where this drug is currently used but also in those where CAPE is proposed to be efficacious.

Future Studies

Future studies will involve examining the efficacy of CAPE chemoradiotherapy in other glioma and PAC xenografts besides U87MG and BxPC-3. Determining the varying degrees of efficacy in different cell lines will be useful in isolating the factors that affect response to CAPE or CAPE treatment regimens. Additionally, the availability of low-density microarrays will allow the determination of global gene expression changes among different treatment groups. This determination will permit the screening of hundreds of genes to ultimately identify a select number of genes that may be particularly relevant to the response to, metabolism of, or resistance to CAPE treatment.

An improvement on the currently used xenograft models can also be made in the future. Orthotopic xenograft mouse models could be used as valuable tools for improving the understanding and control of metastatic disease. These models allow spontaneous metastasis and are therefore more representative of metastatic disease relative to our contralateral xenograft model. Additionally, fluorescent orthotopic xenograft animal models can be developed (e.g., using Renilla green fluorescent protein) that would allow sensitive and quantitative detection of metastases.

The proliferative and chemosensitization effects of abscopal XRT warrant further examination. Initially, microfluidic cards will permit the evaluation of multiple genes that are involved in chemotaxis, adhesion, and proliferation of tumors and that may be responsible for the proliferative effects of XRT on contralateral tumors. Similarly, this method could help identify markers involved in the sensitization of contralateral tumors when chemotherapy (CAPE) is introduced. The introduction of proper controls (e.g., irradiation of a limb lacking tumor cells) will also help clarify the role of abscopal XRT.

As mentioned, in-depth studies of the regulation of both TP and DPD (and other indicators of response to CAPE that may emerge) are also needed. For example, the determination of epigenetic changes such as methylation that affect gene expression is an active area of investigation. The elucidation of cytokine effects on not just transcription but transcription synthesis, protein synthesis, and degradation rates will provide additional information on the mechanisms of TP regulation by cytokines. Nuclear run-on assays may be particularly useful in this respect because they can be used to assess whether changes in mRNA are a result of transcription, subsequent RNA degradation, or transport. Additionally, supershift experiments will clarify the specific transcription factors implicated in the regulation of the TP promoter. Furthermore, the examination of RNA stability factors, and their involvement with the TP 3'UTR will aid in defining the role of the TP 3'UTR in causing mRNA stability.

A final point is that clinical evidence for the potential efficacy of a CAPE chemoradiotherapy regimen in GBM and PAC patients is still needed. One of the principal aims in Phase I clinical trials is to examine maximum tolerated doses and dose-limiting toxicities; therefore, subsequent trials achieving statistical significance will reveal whether CAPE-XRT combinations could represent an effective treatment regimen for these patients. The evidence we currently have seems to suggest that this combination would indeed be effective.

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APPENDIX A

INSTITUTIIONAL REVIEW BOARD FOR HUMAN USE APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office of Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and the approval period is for three years. The Assurance number is FWA00005960.

Principal Investigator:	SAIF, MUHAMMAD W
Co-Investigator(s):	
Protocol Number:	**F020520005**
Protocol Title:	*UAB 0133 - A Phase I/II Open Label Study of Capecitabine With Concurrent Radiotherapy for Patients With Locally Advanced, Unresectable Pancreatic Cancer*

The IRB reviewed and approved the above named project on 3/9/2005. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received FULL COMMITTEE review.

IRB Approval Date: 3/9/2005

Date IRB Approval Issued: 3/16/05

Identification Number: IRB00000726

HIPAA Waiver Approved?: No

Ferdinand Urthaler, M.D.
Chairman of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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BIRMINGHAM AL 35294-0104

OMB No. 0990-0263
Approved for use through 07/31/2005

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Department and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule.

Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER: _____	
4. Title of Application or Activity Biomarker Analysis in Pancreatic Cancer Patient Samples		5. Name of Principal Investigator, Program Director, Fellow, or Other JOHNSON, MARTIN R

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960 the expiration date 1/24/06 IRB Registration No. IRB00000725

[ ] This Assurance, on file with *(agency/dept)* _____ covers this activity.
Assurance No. _____, the expiration date _____ IRB Registration/Identification No. _____ *(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status. Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph _____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting) _____ or [X] Expedited Review on (date) 03-03-05
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments
Please note: UAB IRB Protocol Number is X0301220G2
Protocol subject to Annual continuing review
HIPAA Waiver Approved?: Yes
IRB Approval Issued: 03-03-05

Title
Biomarker Analysis in Pancreatic Cancer Patient Samples

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No *(with area code)* (205) 934-1301 13. Email smoore@uab.edu	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
14. Name of Official Marilyn Doss, M.A.	15. Title Vice Chair, IRB
16. Signature Marilyn Doss	17. Date 3-3-05

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office of Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and the approval period is for three years. The Assurance number is FWA00005960.

Principal Investigator: DIASIO, ROBERT B

Co-Investigator(s):

Protocol Number: X001206001

Protocol Title: *Biomarker Analysis in Patient Samples*

The IRB reviewed and approved the above named project on 11-8-04. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 11-8-04

Date IRB Approval Issued: 11-9-04

HIPAA Waiver Approved?: No

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

The Institutional Review Board for Human Use (IRB) has an approved Multiple Project Assurance with the Department of Health and Human Services and is in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on January 1, 1999 and the approval period is for five years. The Assurance number is M-1149.

Principal Investigator: DIASIO, ROBERT B
Co-Investigator(s):
Protocol Number: X000830002
Protocol Title: *Use of Molecular Markers to Predict Responsiveness to Xeloda*

The IRB reviewed and approved the above named project on 4/24/02. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 4/24/02

Date IRB Approval Issued: 04/25/02

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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OMB No. 0925-0418
Approved for use through 01/31/2001

Protection of Human Subjects
Assurance Identification/Certification/Declaration
(Common Federal Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the common rule. See section 101(b) the common rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the common rule. Institutions with an assurance of compliance that covers the research to be conducted or file with the Department, Agency or the Department of Health and Human Services (HHS) should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency. Institutions which do not have such an assurance must submit an assurance and certification of IRB review and approval within 30 days of a written request from the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
☐ ORIGINAL ☐ FOLLOWUP ☐ EXEMPTION	☐ GRANT ☐ CONTRACT ☐ FELLOWSHIP ☐ COOPERATIVE AGREEMENT ☐ OTHER:	

4. Title of Application or Activity	5. Name of Principal Investigator Program Director, Fellow or Other
Role of Oncogenes/Growth Factors in Human Brain Tumor Growth (Molecular and Cellular Biology of Human Gliomas); (Engineered Herpes Simplex Viruses for	GILLESPIE, GEORGE YAN

6. Assurance Status of this Project *(Respond to one of the following)*

☒ This Assurance, on file with Department of Health and Human Services, covers this activity
Assurance identification no. M-1149 IRB certification no. 01

☐ This Assurance, on file with *(agency/dept)* _____ covers this activity.
Assurance identification no. _____ IRB identification no. _____ *(if applicable)*

☐ No assurance has been filed for this project. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

☐ Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b) paragraph

7. Certification of IRB Review (Respond to one of the following if you have an Assurance on file)

☒ This activity has been reviewed and approved by the IRB in accordance with the common rule and any other governing regulations or supports on (date) 6/21/01, by: ☐ Full IRB Review or ☒ Expedited Review

☐ This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the common rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments

Please note: UAB IRB Protocol Number is X9806090C3

Protocol subject to Annual continuing review.

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed and certification will be provided	10. Name and Address of Institution
	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
11. Phone No. *(with area code)* (205) 934-3789	12. Fax No. *(with area code)* (205) 934-1301
13. Name of Official Marilyn Doss, M.A.	14. Title Vice Chair IRB
15. Signature Marilyn Doss	16. Date 6/21/01

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APPENDIX B

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

MEMORANDUM

DATE: January 8, 2004

TO: Robert B. Diasio, M.D.
VH-101 0019
FAX: 934-8240

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use COmmittee (IACUC) on January 8, 2004.

Title: Pre-Clinical Rationale for a Dual Modality Treatment Utilizing Capecitabine and Irradiation to Treat Breast Carcinoma Metastatic to the Brain: A Pharmacogenomic Approach

Sponsor: Susan G. Komen Breast Cancer Foundation

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

MEMORANDUM

DATE: July 2, 2002

TO: Martin R. Johnson, Ph.D.
WTI-558
FAX: 975-5850

FROM Gilbert J. Grubbs, Ph.D., Chairman
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on June 18, 2002.

Title of Application: A Rational Pharmacogenomic Approach for Treating Glioblastoma Multiforme

Fund Source: American Cancer Society

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
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GRADUATE SCHOOL
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DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Carmelo Blanquicett

Graduate Program Pharmacology and Toxicology

Title of Dissertation A Parmacogenomic Approach to Treating Brain and Pancreatic Cancers Using Capecitabine Chemoradiotherapy

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
Robert B. Diasio	**, Chair**	
Donald J. Buchsbaum		
Martin R. Johnson		
William Booth Parker		
Jeffrey B. Smith		
Ruiwen Zhang		

Director of Graduate Program

Dean, UAB Graduate School

Date April 21, 2005