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Prognostic significance of xCT polymorphisms and expression in patients with advanced pancreatic cancer treated with chemotherapy

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Prognostic significance of xCT polymorphisms and expression in patients with advanced pancreatic cancer treated with chemotherapy

A

THESIS

Presented to the Faculty of the University of Texas

Health Science Center at Houston

and

M. D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in partial fulfillment of the requirements

for the Degree of

MASTER OF SCIENCE

by

Tzu-chuan Jane Huang, MD

Houston, Texas

December 2011

Dedication

This thesis is dedicated to those who made this possible:

My parents, Ping-yuan and Yao-wen Huang, for instilling in me the belief that hard work and perseverance bring limitless possibilities. They have encouraged me to go after my dreams. I could not have asked for more loving and supportive parents.

My brothers, William and Kevin Huang, for supporting me and giving me perspective through challenging times. Growing up alongside them was very special.

My husband, Philipp Torres, for believing in me with his unyielding support and love. He has been my closest friend and has kept me grounded through all the experiences of medical school, residency and fellowship training, and working as physicians. Having him as a life partner makes everything better.

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Prognostic significance of xCT polymorphisms and expression in patients with advanced pancreatic cancer treated with chemotherapy

Tzu-chuan Jane Huang, MD

Supervisory Professor: Milind Javle, MD

The plasma membrane x_c^- cystine/glutamate transporter mediates cellular uptake of cystine in exchange for intracellular glutamate and is highly expressed by pancreatic cancer cells. The *xCT* gene, encoding the cystine-specific xCT protein subunit of x_c^-, is important in regulating intracellular glutathione (GSH) levels, critical for cancer cell protection against oxidative stress, tumor growth and resistance to chemotherapeutic agents including platinum. We examined 4 single nucleotide polymorphisms (SNPs) of the *xCT* gene in 269 advanced pancreatic cancer patients who received first line gemcitabine with or without cisplatin or oxaliplatin. Genotyping was performed using Taqman real-time PCR assays. A statistically significant correlation was noted between the 3' untranslated region (UTR) *xCT* SNP rs7674870 and overall survival (OS): Median survival time (MST) was 10.9 and 13.6 months, respectively, for the TT and TC/CC genotypes ($p = 0.027$). Stratified analysis showed the genotype effect was significant in patients receiving gemcitabine in combination with platinum therapy ($n = 145$): MST was 10.5 versus 14.1 months for the TT and TC/CC genotypes, respectively ($p = 0.013$). The 3' UTR *xCT* SNP rs7674870 may correlate with OS in pancreatic cancer patients receiving gemcitabine and platinum combination therapy. Paraffin-embedded core and

surgical biopsy tumor specimens from 98 patients with metastatic pancreatic adenocarcinoma were analyzed by immunohistochemistry using an xCT specific antibody. xCT protein IHC expression scores were analyzed in relation to overall survival in 86 patients and genotype in 12 patients and no statistically significant association was found between the level of xCT IHC expression score and overall survival ($p = 0.514$). When xCT expression was analyzed in terms of treatment response, no statistically significant associations could be determined ($p = 0.908$). These data suggest that polymorphic variants of *xCT* may have predictive value, and that the xc- transporter may represent an important target for therapy in pancreatic cancer.

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COMMONLY USED ABBREVIATIONS

◦C	Centigrade
CA19-9	Carbohydrate antigen 19-9
Chr	Chromosome
CI	Confidence interval
DAB	Diaminobenzidine
dH20	Distilled water
DNA	Deoxyribonucleic acid
ECOG	Eastern cooperative oncology group
ERCC1	Excision repair cross-complementation group 1
Ex	Exon
FDA	Food and drug administration
FFPE	Formalin fixed paraffin embedded
FOLFIRINOX	5-fluorouracil, leucovorin, irinotecan, oxaliplatin
GSH	Glutathione
H202	Hydrogen peroxide
HR	Hazard ratio
IHC	Immunohistochemistry
MAF	Minor allele frequency
MST	Median survival time
mRNA	Messenger ribonucleic acid
µL	Microliter
µm	Micrometer

MMR	Mismatch repair
NCI	National Cancer Institute
ng	Nanogram
OS	Overall survival
PBS	Phosphate buffered solution
PD	Progressive disease
PFS	Progression free survival
PR	Partial response
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Stable disease
SNP	Single nucleotide polymorphism
System xc-	Cystine/Glutamate antiporter
UGT	Uridine diphosphate glucuronosyltransferase
UTMDACC	University of Texas MD Anderson Cancer Center
UTR	Untranslated region
xCT	Cystine specific subunit of system xC-

Chapter 1

INTRODUCTION

Pancreatic Adenocarcinoma

In 2011, approximately 44,030 new pancreatic adenocarcinoma cases will be diagnosed in the United States, with 37,660 estimated resulting deaths [1]. Although accounting for only 3% of all new cases of cancer, pancreatic cancer continues to be the fourth leading cause of cancer-related death for both men and women in the United States [1]. The diagnosis of pancreatic adenocarcinoma portends a poor prognosis with a mortality rate nearly matching its incidence [1]. This is a highly aggressive cancer that causes substantial disease-related morbidity, metastasizes early in its natural history, and exhibits treatment resistance [2]. While surgery is the only potentially curative therapeutic modality when a microscopic margin negative resection is achieved, only 15–20% of patients have resectable pancreatic cancer. Of these resected early stage pancreatic adenocarcinomas, the 5-year survival rate is only 20% due to eventual development of metastases [3]. Despite advances in conventional multimodality approaches of surgery, radiation and chemotherapy, mortality rates of pancreatic adenocarcinoma have remained relatively unchanged for the last two decades and contribute to a five year overall survival rate of less than 4% [2, 4]. For this reason, understanding the contribution of molecular mechanisms to disease natural history and identifying novel molecular markers are important goals in the management of this cancer.

Molecular Heterogeneity in Pancreatic Cancer

Progressive accumulation of both inherited and acquired mutations leads to the molecular heterogeneity of pancreatic adenocarcinoma [5]. This genetic heterogeneity can be considered broadly in terms of three main molecular events: oncogenic activation driven by genetic mutations, inactivation of tumor suppressor genes, and inactivation of genome maintenance genes critical to cellular repair mechanisms [6]. The extensive inter-tumor genetic variability existing from individual to individual gives rise to multiple permutations of genetic changes. Jones et al. demonstrated this high complexity of the pancreatic cancer genome by determining each cancer has an average of 63 somatic alterations, most of which are point mutations [7]. However, the deregulation of 12 core biological regulatory processes or pathways underlie these large numbers of functional genetic alterations in the majority of pancreatic tumors [7]. Due to this considerable degree of genetic heterogeneity coupled with disappointing survival outcomes with current available therapies, patients with pancreatic adenocarcinoma are in particular need of a personalized approach to cancer therapy.

Challenges of Current Therapy of Pancreatic Cancer

The majority of patients present with unresectable late stage locally advanced or metastatic disease (stage III or IV) that precludes cure by radiotherapy or surgery and have tumors highly resistant to most chemotherapies [8, 9]. Despite the role of cytotoxic chemotherapy as the mainstay of pancreatic cancer therapy, most patients with pancreatic cancer will eventually progress and develop distant metastatic

disease. For patients with advanced disease, mono- or combination systemic chemotherapy that is gemcitabine or fluoropyrimidines-based currently is the standard of care. In metastatic disease, treatment with gemcitabine is associated with symptom improvement in more than 20% of patients and offers a slight survival benefit (5.65 versus 4.4 month overall median survival) when compared to patients treated with 5-fluorouracil [10]. Drug resistance has hindered gains in survival and kept beneficial effects largely confined to symptom palliation [11].

Role of Platinum Analogues in Pancreatic Cancer

Combination chemotherapy in pancreatic cancer has resulted in improved outcomes for patients possessing a good functional performance status [12]. The combination of gemcitabine and a platinum analogue has become first line standard care treatment of advanced pancreatic cancer patients based on results from a meta-analysis of randomized trials [13]. While the combination of gemcitabine with cisplatin has not yielded significant survival benefit over single agent gemcitabine in Phase III studies [14-16], individuals with certain heritable forms of pancreatic cancer may exhibit particular disease sensitivity to platinum agents and benefit with improved responses to this regimen [17-19]. When compared to gemcitabine monotherapy, the addition of oxaliplatin to gemcitabine in advanced pancreatic cancer patients has demonstrated clinically significant advantages of superior response rates, median progression-free survival, and disease-related symptom palliation [20]. More recently, the drug regimen of 5-fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) resulted in more promising results with

significant overall survival advantage (10.5 months versus 6.9 months, $p>0.001$) when compared with single agent gemcitabine. FOLFIRINOX is now considered the preferred frontline treatment regimen for good performance status patients with unresectable or metastatic pancreatic adenocarcinoma [12]. The improved clinical outcomes prompting increased use of platinum analogues in the frontline and salvage settings warrant further study of underlying molecular pathways particular to platinum resistance.

Platinum Resistance

Gaining further understanding of drug-resistance mechanisms is essential to improving the treatment outcome of patients with pancreatic cancer, as the identification of novel targets could lead to the development of therapeutic strategies and provide valuable information to optimize patient selection for particular drugs. Studies in pancreatic cancer have shown that acquired and intrinsic drug resistance is mediated by multiple mechanisms within or outside the cell or at the cell membrane resulting from the dysregulated expression of proteins regulating cell proliferation, death, transport and metabolism of drugs, and DNA repair [21].Two key DNA repair pathways of nucleotide excision repair and mismatch repair are thought to be primary drivers determining sensitivity to cisplatin and its analogues [22]. *In* vitro studies in ovarian and testis tumor cell lines demonstrate that deficiency of the excision repair cross-complementation group 1 (ERCC 1) protein, which is required for the excision of damaged DNA, interrupts the highly conserved nucleotide excision repair DNA repair pathway and leads to

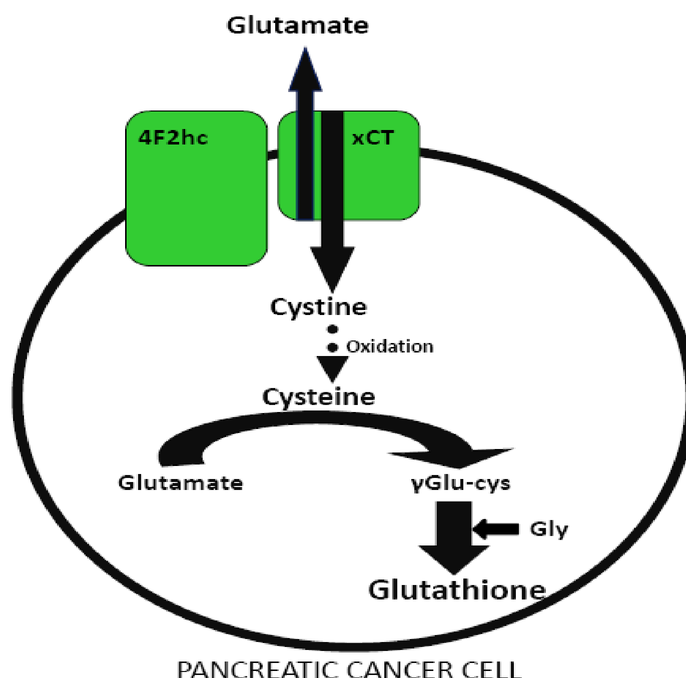
decreased cisplatin sensitivity [23, 24]. Mismatch repair pathway (MMR) deficiency applies to the platinum agents cisplatin and carboplatin. Inherited genetic changes or acquired defects due to epigenetic silencing results in failure of repair proteins to recognize mismatched or unmatched DNA base pairs or insertion-deletion loops and, thus, inability to correct platinum induced DNA damage [25-28]. As a result, cells become resistant to cisplatin and carboplatin, continuing to proliferate despite sustaining treatment-generated DNA damage. Oxaliplatin shows only partial cross resistance to cisplatin in preclinical studies [29]. In addition to causing DNA damage, preclinical data suggests that cisplatin and oxaliplatin activate cell death through generation of reactive oxygen species (ROS); another mechanism of both cisplatin and oxaliplatin resistance results from generation of the intracellular antioxidant molecule, glutathione (GSH), which mediates elimination of drug-induced ROS [29, 30, 31]

Cystine-Glutamate Antiporter (System xc-)

The plasma membrane cystine/glutamate antiporter (system xc-) is an amino acid transport system consisting of a light protein subunit with specificity for cystine, xCT (*SLC7A11* gene), that is coupled to a ubiquitous non-specific heavy protein subunit found in other transporters, 4F2hc (*SLC3A2* gene) [32]. In human tissues and cells, system xc- expression has mainly been demonstrated in the pancreas, along with other cells from the brain, stromal and immune system [33]. A variety of cancer cells also express system xc-, including prostate cancer, lymphoma, glioma, lung cancer and pancreatic cancer [34-37]. xCT transports extracellular cystine

(oxidized form of cysteine) in to cells in exchange for the efflux of glutamate in an obligate relationship at a 1:1 ratio (Fig 1) [33]. Once inside the cell, the dimeric amino acid cystine rapidly reduces to cysteine, the rate-limiting substrate for glutathione biosynthesis [38]. GSH is a tripeptide thiol of glutamate, cysteine and glycine, functioning as a major protective redox-regulatory molecule against free radical induced cellular damage, mutagens, toxins, and drugs [39, 40]. GSH is also co-factor for antioxidant enzymes and, thus, is a major reactive oxygen species scavenger [41]. Thus, xCT plays a critical role protecting cells by counteracting conditions of oxidative stress through its regulation of cystine influx and hence intracellular GSH levels and contributing to cellular detoxification of chemotherapy [42]. This antiporter keeps the redox relationship between extracellular cystine and cysteine in equilibrium [33, 43].

Fig. 1. System xc-: Cystine/Glutamate Antiporter and the Pancreatic Cell.



Pharmacogenomics & Personalization of Cancer Therapy

Pharmacogenomics is the study of how an individual's genotype influences the body's response to drugs and can give insight to drug efficacy in specific patient populations. The term comes from the words pharmacology and genomics and represents the intersection of both disciplines. Germline single nucleotide polymorphisms (SNPs) in the gene encoding the detoxification enzyme, uridine diphosphate glucuronosyltransferase (UGT) 1A1, have been linked to increased toxicity to the drug irinotecan [44]. Patients homozygous for the*UGT1A1*28* allele metabolize the excretion of the irinotecan metabolite, SN-38, more slowly and are at increased risk for neutropenia following this therapy [45, 46]. With FDA approval of a test to identify individuals carrying this mutation, identification of this genetic variant illustrates the predictive possibilities of SNPs. These techniques hold promise for individualizing and optimizing treatments for patients with pancreatic cancer.

xCT and Chemoresistance

Given the role of xc- system in the maintainance of intracellular GSH, it may play an important role in cellular resistance to cisplatin, oxaliplatin and other chemotherapeutic agents. System xc- has been demonstrated to contribute to chemotherapy resistance in preclinical studies, with resistance of tumor cells to anticancer drugs correlated with increased GSH levels. The level of xCT expression can be induced in conditions of oxidative stress and seems also to play a role in cancer cell proliferation [37]. Microarray gene expression analysis of

system xc- in 60 human cancer cell lines used by the National Cancer Institute for drug screening (NCI-60) demonstrated that the level of xCT expression is positively correlated with sensitivity of tumor cells to anticancer drugs, with its inhibition compromising both cellular redox defense and resistance to multiple drugs [47]. Lo et al demonstrated that the highly chemotherapy resistant pancreatic cell line PANC-1 expresses higher xCT expression in comparison to pancreatic cell lines MIAPaCa-2 and BxPC-3 [37]. Similar findings of higher xCT expression correlated with cisplatin resistance also has been demonstrated in resistant human ovarian cancer and colon cancer cell lines. Further, data from *in vitro* systems have shown that inhibition of xCT restores sensitivity to gemcitabine [49]. An understanding of the pharmacology including the pharmacogenomics of the xc- system is therefore worthy of further study.

From these preclinical observations, the following hypotheses are made: 1) genetic variations of the cystine/glutamate transporter are associated with overall survival and response to chemotherapy in patients with advanced pancreatic cancer treated with gemcitabine +/- cisplatin and 2) high xCT expression in pancreatic cancer tissue is associated with a lower overall survival in patients with unresectable advanced pancreatic cancer.

Chapter 2

METHODS

Study Population

Patients were initially identified from patients participating in a case-control study of pancreatic cancer conducted at The University of Texas MD Anderson Cancer Center (Houston, Texas) from 1999 through 2009. The study was approved by the institutional review board of University of Texas MD Anderson Cancer Center. The eligibility criteria included patients having: a diagnosis of a primary pancreatic ductal adenocarcinoma that was pathologically confirmed at MD Anderson, gave consent to blood donation, no prior therapy received, and who received first-line single-agent gemcitabine or gemcitabine in combination with cisplatin or oxaliplatin treatment at MD Anderson. All patients signed an informed consent for medical record review and provided a sample of whole blood by peripheral phlebotomy. Clinical, pathology, and radiographic records of the selected patients were then reviewed using the institutional electronic medical records database (ClinicStation™) to confirm their diagnosis and disease stage. Available outside records which had been digitally scanned into the system were also reviewed. Patients who were seen only at their initial visit without subsequent follow up visits at MD Anderson were excluded. Patients who had pancreatic neuroendocrine tumors were also excluded.

Clinical Data Collection

Clinical information was retrieved by reviewing patients' medical records and included gender, age at diagnosis, date of pathologic diagnosis, clinical tumor stage (resectable, locally advanced, metastasized, and unstaged), serum carbohydrate antigen 19-9 (CA19-9) values (unit/mL) at diagnosis, patient performance status, chemotherapy received in the first-line setting at the time of metastasis and date of death or last follow-up. Overall survival duration was calculated from the time of pathologic diagnosis to the date of death or last follow-up. The clinical information was double-checked by different researchers. Clinical response to chemotherapy was assessed by evaluation of radiographic reports and determination by the treating physicians as documented in clinical progress notes. The clinical endpoint was overall survival and treatment response.

Specimen Collection and DNA Extraction

Peripheral lymphocytes were collected from freshly drawn blood by Ficoll–Hypaque (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation and stored at –80 °C. The FlexiGene DNA kit (QIAGEN, Valencia, CA) and the Maxwell 16 automated system (Promega, Madison, WI) were used to extract DNA, which was stored at 4 °C.

Genotyping

Four functional SNPs located in the coding region (synonymous) or the untranslated region (UTR) of the SLC7A11 gene were selected. The four SNPs included three

synonymous SNPs of rs35701885, rs4479754, rs6838248 and one 3'-UTR (untranslated region) SNP rs7674870. The gene, chromosome (Chr) location, function, amino acid changes and minor allele frequency (MAF) of the 4 SNPs evaluated in this study are summarized in Table 1.

Table 1. SNPs evaluated

Gene	Chr	dbSNP rs#	Chr Position	Genomic Systematic	Proteomic Systematic	Function	Wild type allele	Variant allele	MAF *
SLC7A11	4q28-q32	rs7674870	139308913	Ex12+3709T>C	3'UTR	3' UTR	A	C	0.35
		rs35701885	139323865	Ex8+45G>A	P320P	Synonymous	G	A	0.04
		rs4479754	139319822	Ex11-2G>A	S481S	Synonymous	A	G	0.08
		rs6838248	139359944	Ex5+26C>G	Ex5+26C>G	Synonymous	C	G	0.331

* Allele frequencies obtained from the national center for biotechnology information dbSNP cancer database.

Genotyping was performed using the Taqman 5′ nuclease assay. Primers and TaqMan MGB probes were provided by TaqMan SNP Genotyping Assay Services (Applied Biosystems, Foster City, CA). PCR was performed in a 5-μL total volume consisting of TaqMan Universal PCR Master Mix, 20 ng of genomic DNA (diluted with dH_2O), and TaqMan SNP genotyping assay mix. Alleles were discriminated by running end point detection using an ABI Prism 7900HT sequence detection system and SDS 2.3 software (Applied Biosystems, Foster City, CA). Approximately 10% of samples were analyzed in duplicate, and inconsistent data were excluded from final analysis.

Immunohistochemistry

In addition to the above described cohort, patients with metastatic pancreatic adenocarcinoma evaluated at MD Anderson were also identified from their medical records. Pathology records of these patients were then used to determine the availability of patient tissue samples. For patients who had a biopsy or surgical procedure at MD Anderson, their formalin fixed paraffin embedded tissue samples were requested and obtained from the pathology file room. For patients treated at MD Anderson who received a biopsy or surgery at other institutions, formalin fixed paraffin embedded samples from these patients were also requested and obtained from outside hospitals. All patients had their pathologic samples confirmed by a pathologist at MD Anderson.

Formulin fixed paraffin-embedded (FFPE) core and surgical biopsy tumor specimens from 98 patients with metastatic pancreatic adenocarcinoma were analyzed by immunohistochemistry (IHC). Tissue samples were cut to 4-5 μm thick sections using an automated Leica RM2255 rotary microtome and mounted on silanized positively charged slides.

FFPE tissue histology sections were deparaffinized, hydrated and incubated for 120 minutes. Antigen retrieval was performed using steam preheated to 92-97°C and submerged in 0.01 M Citrate at pH 6. Samples were cooled for 20 minutes at room temperature and then washed in 4X PBS for 15 minutes. Peroxide blocking was done with 3% H_2O_2 in PBS at room temperature for 10 min, followed by washing in

4X PBS for 15 minutes, and then blocked with normal serum for 20 minutes at room temperature.

Slides were incubated with a rabbit primary polyclonal antibody against xCT (Novus Biologicals, LLC, Littleton, CO) at 1:100 dilution at 4℃ overnight and then probed at room temperature for 60 minutes with the secondary antibody Vectastain Elite (Vector Laboratories, Burlingame, CA). Samples were washed for 5 minutes using 3X PBS and incubated with ABC reagent for 30 minutes. Staining was developed with 0.05% 3,3'-Diaminobenzidine (DAB) substrate (Invitrogen, a division of Life Technologies Corporation, Carlsbad, CA) and counterstained with hematoxylin.

Grading of IHC slides

IHC scores were calculated by the product of intensity and extent of xCT expression by visualization of 6 fields (staining-intensity X percentage of staining-extent). The intensity of tumor staining for xCT was quantified using a four value intensity score that was categorized as: absent (score 0, non-expressed), very weak (score 1, slightly expressed), weak (score 2, expressed), or strong (score 3, highly expressed). Detection of positive staining in ≤50% or >50% resulted in a respective score of 1 or 2 for staining extent. Cells with a final score ≥2 were considered positive for protein expression in cytoplasmic (membrane) staining.

Survival Measurements

Overall survival was measured from the date of diagnosis to the date of death or last follow-up. Dates of death were obtained and confirmed using at least one of the following three methods: Social Security Death Index (www.deathindexes.com/ssdi.html), inpatient medical records, and the MD Anderson tumor registry.

Statistical Analysis

The genotype distribution was tested for Hardy-Weinberg equilibrium with the goodness-of-fit X^2 test. The heterozygous and homozygous genotypes were collapsed in the analysis if the frequency of the homozygous mutant was very low or if the homozygous and heterozygous genotypes had the same direction of effect, e.g., both had reduced survival time compared to the referent group.

Median survival times (MST) were calculated for all patients. Kaplan-Meier method was used for survival analyses, groups were compared using log-rank test. Hazard ratios and 95% confidence intervals (95% CI) were estimated using univariable or multivariate Cox proportional hazard models. Known or potential prognostic clinical factors (CA 19-9, race, performance status) were included in the multivariate model when appropriate. All statistical testing was conducted with SPSS software, version 17.0 (SPSS), and statistical significance was defined as $p < 0.05$. All tests were two sided. The false-positive report probability for the observed statistically significant association was estimated using the methods described by Wacholder et al [50]. A

prior probability of 25% was considered appropriate given the biologic plausibility and previous established biostatistical evidence in support of such an association. The false-positive report probability value for noteworthiness was set as 0.2.

Immunohistochemistry

xCT protein IHC expression scores were analyzed in relation to overall survival and response to treatment of the patients. Mean differences of groups were analyzed using the one factor ANOVA test. Dichotomous scoring, with 0 representing expression scores ≤ 3 and 1 representing expression scores > 3, was also used to evaluate the association between the protein expression and overall survival of the patients. Kaplan-Meier method was used for survival analyses, groups were compared using log-rank test. Cox proportional hazard regression models were fitted to determine the association between xCT IHC expression and overall survival. Prognostic variables entered into the model included ECOG performance status, CA 19-9, and stage.

Chapter 3

RESULTS

SNP Analysis.

Patient characteristics and clinical predictors

The patient characteristics are summarized in Table 2. There were no significant differences in overall survival by age, sex, or race of the 269 patients evaluated. Of them, 148 (55%) patients had metastatic disease (stage 4) and 121 (45%) patients had locally advanced pancreatic adenocarcinoma (stage 3).

Table 2. SNP Analysis: Patient characteristics (Total n = 269).

Variable	Number of Patients (n)	Percentage (%)
Age		
≤50	39	14.5
51-60	67	24.9
61-70	102	37.9
>70	61	22.7
Sex		
Male	159	59.1
Female	110	40.9
Race		
White	242	90
Hispanic	14	5.2
Black	10	3.7
Asian	3	1.1
Stage		
3	121	45
4	148	55

We identified three prognostic factors that were significantly associated with improved survival outcomes, which include an earlier disease stage, a lower CA 19-9 tumor marker, and a better performance status (Table 3). This analysis was consistent with previously identified independent prognostic factors in advanced pancreatic cancer published in the literature, demonstrating our patients are a representative cohort.

Table 3. SNP Analysis: Clinical Prognostic Factors (n=269).

	N. patients	No. deaths	MST (months)	95% CI	p value
Stage					0.002
3	121	110	15.7	13.84-17.63	
4	148	138	9.7	7.95-11.45	
***ECOG Performance Status**					0.026
0	29	24	15.7	13.77-17.7	
1	129	121	13.2	11.69-14.77	
2	23	23	10.4	6.94-13.93	
CA 19-9					0.005
<47	35	28	17.8	11.47-24.13	
48-500	93	86	14.1	12.34-15.92	
>500	126	119	11.4	9.65-13.15	

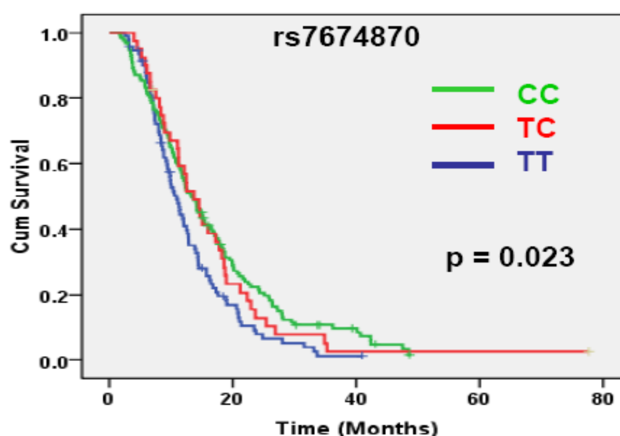
Information was missing from 88 patients.

Genotype and association with OS.

Of the four SNPs evaluated, one showed a significant association with OS, i.e. the 3' UTR xCT gene SNP, rs7874870. As shown in Figure 2, patients having CC and

TC genotypes had a significantly better overall survival than the TT genotype, the median survival time (MST) was 13.7, 13.3, and 10.9 months, respectively (p value = 0.023). We estimated the false-positive report probability of the xCT SNP rs7674870 to be 0.077, given a prior probability of 25%. It is below the threshold of 0.20 indicating noteworthiness.

Figure 2. Kaplan-Meier curve of overall survival in all patients by the rs7674870 genotype. The genotype is indicated by the blue (TT homozygous), red (TC heterozygous) and the green (CC homozygous) lines.



Genotype	n	MST (months)	HR (95% CI)	P value
TC	131	13.3	0.70 (0.53-0.93)	0.023
CC	40	13.7		
TT	93	10.9	1.0	

Because of similar survival, the CC and TC groups were combined for further statistical analysis. This range of overall survival is comparable with the general population of pancreatic cancer patients and further confirms our study population is

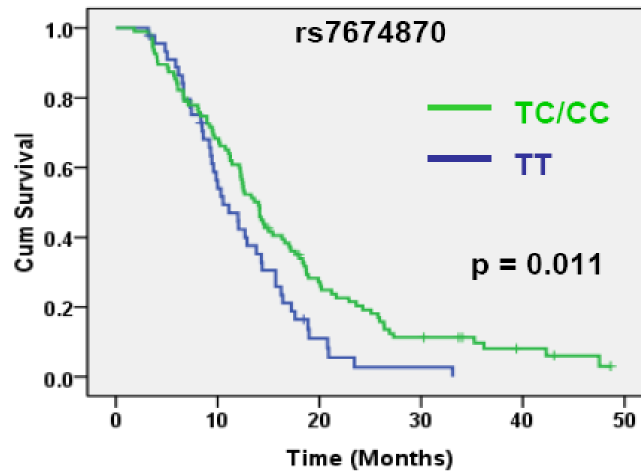
a representative cohort. None of the three synonymous SNPs were found to be significantly associated with OS. The genotype frequencies, MSTs and hazard ratios (95% CI) are shown in Table 4.

Table 4. OS by genotype.

SNP	Number of patients (n)	MST (month)	HR (95% CI)	p value
rs4479754				0.646
AA	260	12.3	1.0	
AG	4	5.9		
GG	1	9.2		
AG/GG			1.2 (0.54-2.66)	
rs6838248				0.990
CC	82	12.7		
CG	113	12.3	1.0	
GG	70	11.1	1.01 (0.85-1.19)	
rs35701885				0.543
GG	200	12.4	1.0	
GA	21	15.1		
AA	0	-		
GA/AA			0.92 (0.59-1.43)	

Stratified analysis showed that this genotype effect remained significant in patients receiving gemcitabine in combination with platinum analogs, with MST of 10.5 months for the TT genotype and 14.1 months for the TC/CC genotypes (p value = 0.011) (Fig 3).

Figure 3. Survival by genotype in patients receiving Gemcitabine + Platinum Therapy

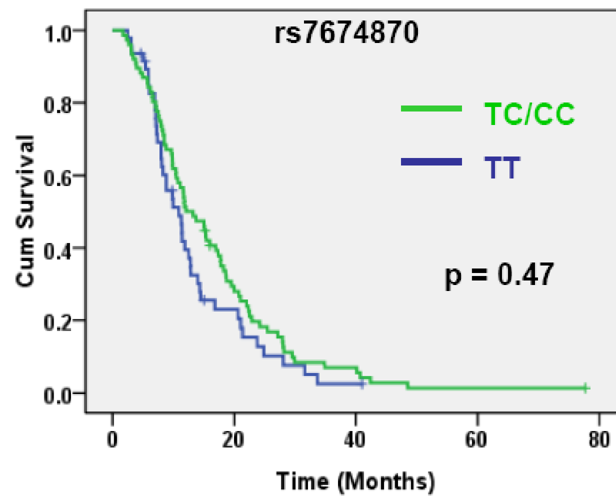


Genotype	n	MST (months)	HR (95% CI)	p value
TC/CC	95	14.1	0.60 (0.40-0.89)	0.011
TT	45	10.5	1.0	

n

The genotype effect was not significant in patients treated with gemcitabine monotherapy, with MST of 10.9 months for TT and 12.0 months for TC/CC genotypes (p value = 0.47) (Fig 4).

Figure 4. Overall survival by genotype in patients receiving first-line gemcitabine monotherapy.



Genotype	n	MST (months)	HR (95% CI)	p value
TC/CC	76	12	0.99 (0.63-1.57)	0.47
TT	47	10.9	1.0	

None of the three synonymous SNPs (rs4479754, rs6838248, and rs35701885) were found to be significantly associated with OS (Table 4).

Immunohistochemistry

98 patient samples were available for evaluation by immunohistochemistry (Table 5). All patients had metastatic pancreatic adenocarcinoma. There were no significant differences in overall survival by age, sex, race or IHC expression score of the patients evaluated. Clinical treatment history was available for 86 samples and were eligible for treatment response data analysis. At the time the data were censored, 79.5% of the patient population had died.

Table 5. Immunohistochemistry: Patient Characteristics (n=86)

	Number of Patients (n)	Percentage (%)	HR (95% CI)	p value
Age				0.699
≤50	16	18.6		
51-60	25	29.1		
61-70	31	36		
>70	14	16.3	0.995 (0.971-1.020)**	
Sex				0.729
Male	61	70.9	1.0	
Female	25	29.1	1.111 (0.614-2.007)	
Race				0.102
White	74	86	1.0	
Hispanic	4	4.7		
Black	6	7		
Other	2	2.3		
Non-white			0.715 (0.479-1.069)	
IHC Score*				0.782
Low	36	42.9	1.0	
High	48	57.1	0.924 (0.530-1.614)	
Stage				
4	86	100		

*2 samples were not evaluable after staining
**Continuous variable

There was no statistically significant association between the level of xCT IHC expression score and MST, with 8.8 month MST for high xCT expression and 8.4 month MST for low xCT expression (p=0.514) (Figure 5, Table 6).

Figure 5. xCT immunohistochemical expression correlated with overall survival.

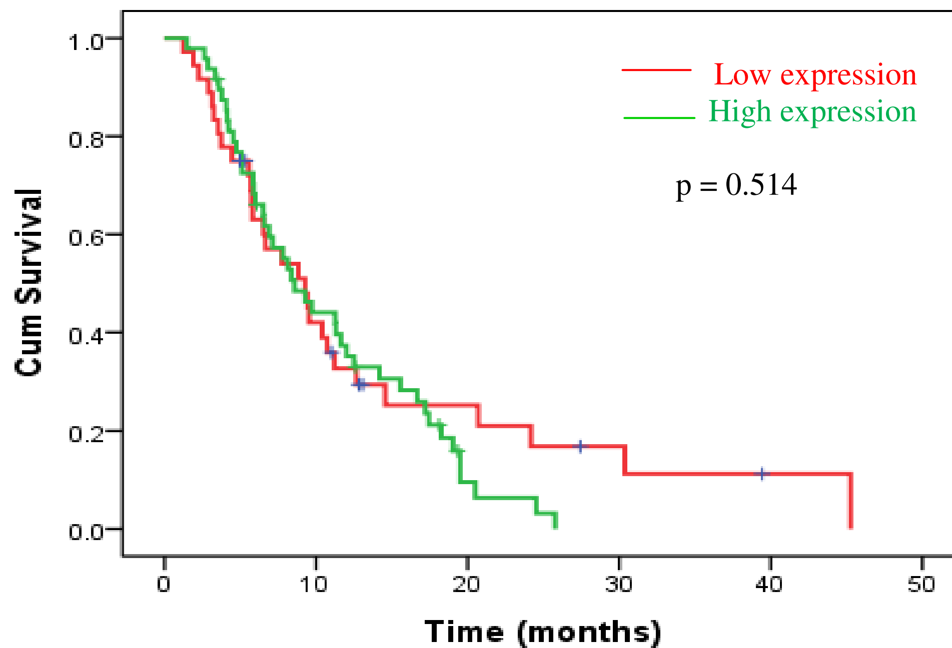


Table 6. xCT IHC and Overall Survival.

	Number of patients	Number of deaths	MST (months)	95% CI	p value
Low IHC Expression	44	35	8.8	5.48-12.18	
High IHC Expression	54	48	8.4	5.92-10.81	
Overall	**98**	**83**	**8.4**	**6.41-10.32**	0.514

When the mean xCT IHC expression staining score was analyzed in terms of patient chemotherapy treatment response, no statistically significant associations could be determined (p = 0.908) (Table7,8). The trend of higher xCT expression, however, is consistent with our hypothesis that higher xCT expression is correlated with aggressive disease course and with our overall survival data indicating a shorter median survival time.

Table 7. Average IHC Score and Chemotherapy Response.

Response	n	Mean	95% CI	p value
Progressive disease	37	3.28	2.76-3.82	
Partial response	25	3.12	2.48-3.76	
Stable disease	16	3.16	2.42-3.91	
Total	**78**	**3.21**	**2.87-3.55**	0.908

Table 8. xCT IHC Expression Correlated to Chemotherapy Response.

Response	**Score $\leq$ 3** n (%)	**Score > 3** n (%)	**Total** n (%)	p value
Progressive disease	15 (40.5)	22 (59.5)	30	
Partial response	11 (44)	14 (56)	25	
Stable disease	8 (50)	8 (50)	16	
TOTAL with response	**34 (43.6)**	**44 (56.4)**	**78**	0.514

Stratified analysis of xCT immunohistochemical expression score and survival by chemotherapy treatment groups of gemcitabine monotherapy and combination chemotherapy with gemcitabine and platinum containing agent did not show any significant associations (Table 9, 10).

Table 9. IHC: Survival Analysis of Patients Treated with Gemcitabine (n=36).

	Number of patients	**Number of deaths**	**MST (months)**	**95% CI**	**p value**
Low IHC Expression	18	14	8.83	2.81-14.86	
High IHC Expression	18	15	8.57	6.05-11.08	
Overall	**36**	**29**	**8.83**	**6.66-11.00**	**0.73**

Table 10. IHC: Survival Analysis of Patients Treated with Gemcitabine and Platinum Agent (n=43).

	Number of patients	**Number of deaths**	**MST (months)**	**95% CI**	**p value**
Low IHC Expression	14	13	7.73	3.30-12.17	
High IHC Expression	29	27	8.37	1.58-15.15	
Overall	**43**	**40**	**7.73**	**4.41-11.06**	**0.98**

Chapter 4
DISCUSSION

Genomic variations may have predictive value in determining response to chemotherapy. In this study, the associations between xCT gene SNP, rs7674870, and clinical outcomes of patients with advanced pancreatic adenocarcinoma were evaluated. To our knowledge, these data are the first to suggest that there is an important role for cystine/glutamate antiporter genes in predicting cisplatin resistance and in the overall survival of patients with advanced pancreatic cancer.

Our results suggest that the 3'UTR rs7674870 TC/CC genotype was significantly associated with OS. The rs7674870 TC/CC genotype remained as a significant predictor for survival after adjusting for all other clinical and genetic factors. Our results indicate the correlation between this genotype and OS of patients receiving combination chemotherapy with gemcitabine and platinum analogs in predicting platinum treatment response.

This study demonstrates that xCT is reliably detectable by immunohistochemistry in human pancreatic cancer tissue. xCT has the functional role of modulating the oxidative environment that is critical to protection of the cancer cell against xenobiotics through its control of cystine uptake and intracellular glutathione levels [51]. Based on preclinical data that system xc- expression is associated with gemcitabine resistance, we expected an association of xCT protein expression with chemotherapeutic response and OS of patients with metastatic pancreatic cancer.

Based upon our results demonstrating a possible role of xCT genotypic variations in cisplatin resistance, we expected the patient cohorts treated with combination therapy of gemcitabine and platinum to have significant differences in survival based on level of xCT expression. Our data did not show any such significant associations.

Many factors may be responsible for these results. *In vitro* preclinical pancreatic cell line and *in vivo* functions may not be similar for xCT, and *in vitro* function may not be recapitulated *in vivo* [52]. Given the discrepancy between *in vitro* and *in vivo* xCT function, the availability of three different xCT loss of function mouse models serve as valuable systems in which to further study xCT function and response to pharmacologic manipulation. In comparison to the technique of Western blotting, immunohistochemistry is limiting as a semi-quantitative assay evaluated by visual assessment which may depend on inter-observer variability and the target of interest. Further, the immunohistochemical expression of the xCT protein may not represent the functional properties of this transporter [37].

We postulated that genetic variations of xCT would manifest in changes of the xCT protein detectable by immunohistochemistry and expected a possible association of this SNP with xCT protein expression. An exploratory analysis in 12 patient samples demonstrated higher xCT expression was associated with reduced survival seen for the TT genotype, which would be consistent with our SNP analysis. Though results from this small patient cohort only trended toward statistical

significance, further genotypic-phenotypic correlative studies may be worthy for future study. Biologically relevant SNPs may not be best studied by protein expression correlative studies as multiple regulatory steps are involved in the pathway from gene to protein. Because synonymous SNPs do not produce altered coding sequences, they are not expected to change the function of the protein encoded. However, a previous study has demonstrated that a synonymous SNP in the MDR1 gene results in a protein product with altered drug and inhibitor interactions [51]. SNPs may also be located at the 3' and 5'-UTR of DNA. While these sequences do not translate into proteins, the 3'UTR may contain sequence motifs crucial for the regulation of transcription, mRNA stability, and cellular location of the mRNA or the binding of microRNA [52]. Further studies of xCT mRNA expression through utilization of Northern blotting and microRNAs, evolutionarily conserved noncoding RNAs that mediate the posttranslational protein modifications by binding to 3' untranslated regions, would be particularly insightful [51].

Given the increasing use of platinum analogues in the frontline setting with the emergence of FOLFIRINOX and continued use of gemcitabine and cisplatin combination chemotherapy in advanced pancreatic cancer, our study is especially relevant. With persistent poor survival outcomes for patients with pancreatic cancer, clearly a need for greater understanding of underlying mechanisms of chemotherapy resistance exists. This would be important not only for discerning disease pathogenesis but also for potentially determining new targets of therapy. xCT may represent a viable novel target in pancreatic cancer.

Several pharmacologic agents that inhibit system xc- exist [54], exerting therapeutic effects primarily by interrupting the antiporter function of cystine uptake into the cell. The resulting state of decreased intracellular cystine levels may lead to cellular growth inhibition and ultimately cause a state of glutathione depletion, thus reducing the ability of the cell to detoxify xenobiotics such as chemotherapy [33]. The established FDA approved anti-inflammatory drug, sulfasalazine, has been studied as an xCT inhibitor in many different in vitro and in vivo systems. In vitro, sulfasalazine causes growth inhibition of the MIAPaCa and PANC-1 pancreatic cancer cell lines [53]. Chung et al. demonstrated that intraperitoneal injection of sulfasalazine pharmacologically inhibits system xc- in glioma cells, reducing glutathione levels in tumor tissue and slowing tumor growth in an intracranial xenograft animal model for human glioma [54]. While sulfasalazine historically having excellent safety profile, a trial evaluating sulfasalazine in the treatment of progressing malignant gliomas had to be terminated early after interim analysis demonstrated significant grade 4 toxicity and patient death on study [55]. Most recently, the synthesis of several sulfasalazine analogues possessing a more favorable pharmacologic profile demonstrate promise in expanding therapeutic options that inhibit system xc- [56].

It is acknowledged that this study has several limitations and that our findings are hypothesis generating due to its exploratory nature. The large number of patients with unresectable advanced pancreatic cancer in this study cohort likely reflects a

referral bias favoring patients with more severe disease at our institution. A selection bias exists due to the retrospective nature of this study. This study population was biologically and clinically heterogenic due to the inclusion of patients with metastatic pancreatic cancer. We evaluated rs7674870 in a patient cohort of 269 patients where 123 received gemcitabine monotherapy and 140 received gemcitabine-platinum (cisplatin/oxaliplatin) combination chemotherapy. xCT immunohistochemistry was performed in 98 patients, of whom 36 was treated with gemcitabine and 43 was treated with gemcitabine-platinum combination. In 12 patients, the association of genotype and immunohistochemical protein expression was analyzed. Hence, the statistical power in terms of prediction and prognosis is limited.

Chemotherapy resistance contributes to poor survival outcomes for patients with advanced pancreatic cancer. Our analyses is one of the first to specifically evaluate the role of xCT polymorphisms to the chemotherapy sensitivity and survival in unresectable pancreatic cancer. In conclusion, genotypes of system xc- xCT transporter genes have potential as predictive biomarkers for cisplatin response and efficacy in unresectable advanced pancreatic cancer. This study establishes that human xCT can be reliably detected and qualitatively scored by immunohistochemistry. Prospective validation of these results in additional datasets and human functional pharmacologic inhibitor studies are needed.

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Vita

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