MOLECULAR PROFILING OF CLINICAL DRUG RESISTANCE

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ABSTRACT

ROSHAWN WATSON:
Molecular Profiling of Clinical Drug Resistance
(Under the direction of Howard McLeod, Pharm.D.)

One of the greatest challenges in oncology is drug resistance. 5-Fluorouracil (5-FU) is the third most commonly used anti-neoplastic, so lack of initial or continued response to 5-FU represents a big clinical problem. Despite its prominence in cancer treatment, the mechanisms for its resistance remain largely undefined. The third leading cause of cancer-related deaths is colorectal cancer for which 5-FU is an essential part its therapeutic backbone. Resistance to 5-FU is a primary cause of treatment failure. Developing models explaining 5-FU resistance is imperative to advancing care.

Quantitative proteomics is a rapidly emerging tool that when combined with functional studies can be valuable for mechanistic elucidation. Our combined modality approach utilizes colorectal tumors that are well-phenotyped with respect to 5-FU exposure (clinical resistance), demographics, and baseline disease characteristics. Expression of critical 5-FU pathway proteins is quantified within both tumors 5-FU exposed and unexposed, expression is then compared, and proteins with differential expression associated with 5-FU resistance are carried forward for functional validation. Then, augmentation of 5-FU sensitivity (IC50) after knockdown of the genes (DUT, UCK2, and DPYD) encoding the differentially expressed proteins was evaluated in

colorectal cancer cell lines. DUT and UCK2 knockdown decreased IC50 by >2-fold in two or more cell lines while DPD knockdown yielded decreased IC50 by >2-fold in one cell line and nearly 2-fold in the others. This mechanistic validation supports overexpression of these targets as a mechanism for 5-FU resistance. Additionally, copy number gains in TYMS occurred 5 times more frequently in exposed compared to unexposed patients, suggesting that TYMS gains is also a mechanism for 5-FU resistance.

This work could have a significant impact on defining mechanisms of drug resistance and designing rationale therapies for resistant patients. This model provides a strategy for not only screening multiple candidates potentially causing resistance but also a method for stratifying samples in a manner that enriches for variations associated with resistance and a means of credentialing these candidates for their putative mechanism.

To my wife, family, and friends

who provided the support system necessary

to make this extraordinary dream a reality!

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Finally, but definitely not least, thank you God for the strength to endure and the conviction to win.

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LIST OF ABBREVIATIONS AND SYMBOLS

5-FU	5-Fluorouracil
AJCC	American Joint Committee on Cancer
AQUA	automated quantitative analysis
BrdU	bromodeoxyuridine
CEA	carcinoembryonic antigen
CEPH	Centre d'Etude du Polymorphisme Humain
CRC	colorectal cancer
Ct	threshold cycle
DAPI	4',6-diamidino-2-phenylindole
DHFU	dihydrofluorouracil
DNA	deoxyribnucleic acid
DPD	dihydropyrimidine dehydrogenase (protein)
dTMP	deoxythymidine monophosphate
dTMP	2V-deoxythymidine-5V-monophosphate
dUDP	deoxyuridine diphosphate
dUMP	2V-deoxyuridine-5V-monophosphate
DUT	deoxyuridine triphosphatase
ELISA	enzyme-linked immunosorbent assay
ERBB2	HER-2/neu
FBS	fetal bovine serum
FdUMP	fluorodeoxyuridine monophosphate
GWA	genome-wide association

HER2	human epidermal growth factor receptor 2
HIPAA	Health Insurance Portability and Accountability Act
HR	Hazard Ratio
IC50	concentration which inhibits biological process by 50%
IHC	immunohistochemical
IRB	Institution Review Board
LOH	loss of heterozygosity
mCRC	metastatic colorectal cancer
mRNA	messenger ribonucleic acid
N/C	nuclear to cytoplasmic protein expression ratios
NCI	National Cancer Institute
NME	non-metastatic cells
PTEN	Phosphatase and Tensin Homolog
qRT-PCR	real time quantitative PCR
RECIST	Response Evaluation Criteria In Solid Tumors
RNAi	RNA interference
RRM1	regulatory unit of ribonucleotide reductase
RRM2	catalytic unit of ribonucleotide reductase
shRNA	short hairpin RNA
SSDI	Social Security Death Index
TMAs	tissue microarrays
TP	thymidine phosphorylase (protein)
TS	thymidylate synthase (protein)

TSER	thymidylate synthase tandem repeat
TYMP	thymidine phosphorylase (gene)
TYMS	thymidylate synthase (gene)
UCK	uridine cytidine kinase

Chapter 1

Introduction

General Introduction

Variability in drug response is one of the most pervasive problems with patient care, especially variability to chemotherapy and immunologic therapy.[1] For instance, despite the addition of novel agents and better optimization of regimens, over 50% of patients undergoing chemotherapy for advanced and metastatic colorectal disease will not receive significant therapeutic benefit.[2-4] Many factors have been shown to influence response to drug therapy including: age, gender, tumor subtype, disease stage, comorbid diseases, overall performance status, pharmacokinetic and pharmacodynamics factors.[5-7] Unfortunately, our knowledge of these factors typically does not translate into development of predictive models for chemotherapy response. Our inability to discriminate between patients who will derive benefit from chemotherapy and those who will not causes non-responders to be exposed to unnecessary toxicity and expenses. Identifying genetic contributors to variability in chemotherapy response allows for the appropriate agents to be selected at the initiation of chemotherapy and when the regimen must be modified. Presently, there are several examples where interindividual differences in patient response have been linked to sequence variation and differences in expression of drug-metabolizing enzymes, drug transporters, and drug targets.[5,6,8] Genetics is believed to account for between 20 to 95% of the variability in drug disposition and effects.[9] Such variability forms the basis for tumor heterogeneity and normal germline differences between cancer patients. It is often a lack of an understanding of patient genomic diversity that makes it particularly difficult to predict chemotherapy response variability.

This challenge has given rise to pharmacogenomics, a branch of personalized medicine that has made progress in dissecting the diverse responses to chemotherapy. Pharmacogenomics is the study of genetic differences underlying interindividual variability in drug responses.[5,10] It includes the studies of variation in RNA expression, somatic mutations, and germline DNA.[11] It aims to identify genetic changes in drug-metabolizing enzymes and other molecules influencing drug activity.[11] Pharmacogenomics is particularly important to oncology because systemic toxicity and unpredictable efficacy often characterize chemotherapy. Additionally, the high costs of chemotherapy make selection of the appropriate agent financially prudent. Incorporating pharmacogenomics into treatment decisions has been shown to improve patient outcomes. This is particularly true for colorectal cancer (CRC). In this chapter, we will discuss CRC, resistance to its treatment, targets associated with its resistance, and the methodologies used to identify the determinants to CRC chemotherapy resistance.

Colorectal Cancer and Resistance

Colorectal cancer is currently the third leading cause of cancer-related deaths in the US.[12] Despite some recent advances, such as the development of new treatments and regimens, nearly all patients with metastatic colorectal cancer develop clinical resistance and eventually stop responding to therapy. This resistance has been well-documented with 5-fluorouracil-based regimens.

5-fluorouracil (5-FU) is the backbone of treatment for advanced and metastatic colorectal cancer, as nearly all patients will receive a 5-FU-containing regimen (Figure 1.1).[13,14] The prominence of 5-FU in metastatic colorectal cancer (mCRC) treatment is

largely a function of its consistent efficacy throughout five decades of use.[15] However, one of the biggest challenges for the management of mCRC is 5-FU treatment failure, especially in patients who initially respond but become resistant. Indeed, the five-year survival of those with mCRC is less than ten percent.[12] Deaths due to chemotherapy-resistant mCRC disproportionately account for its very high mortality rate.[16] Given that 5-FU is the third most commonly used anti-neoplastic agent, its resistance represents a major clinical problem.

Targets Associated With Variable 5-FU Resistance

Understanding the mechanism of action for 5-FU may offer insight into mechanisms of its resistance. 5-FU is metabolized *in vivo* by thymidine phosphorylase ultimately into fluorodeoxyuridine monophosphate (FdUMP, Figure 1.2), which binds TS. TS is the rate-limiting enzyme in pyrimidine nucleotide synthesis, and it inhibits deoxythymidine monophosphate (dTMP) production.[17] Since dTMP is essential for DNA biosynthesis and repair, its depletion results in 5-FU's cytotoxicity.[18] Dihydropyrimidine dehydrogenase (DPD)-mediated metabolism of 5-FU into dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism; up to 80% of 5-FU is metabolized by DPD in the liver.[19]

Due to the importance of 5-FU to the treatment of colorectal and other gastrointestinal cancers, there have been numerous investigations into 5-FU resistance. The primary approach of these previous studies has been to evaluate genes, mRNA, and proteins thought to be important to 5-FU resistance (Figure 1.3).[20-23] Pharmacokinetic-mediated 5-FU resistance primarily consists of inadequate accumulation of 5-FU at the

tumor site, altered 5-FU distribution secondary to the cancer, and increased 5-FU elimination. Altered 5-FU metabolism, such as decreased catabolic activation and increased inactivation of metabolites by a metabolizing enzyme (i.e. TP), can also cause resistance.[21] Additionally, pharmacodynamic contributors to 5-FU resistance are important. Target-associated resistance includes increased levels of 5-FU's target enzyme (thymidylate synthase, TS) or its substrate (dUMP) and thymidylate synthase gene (TYMS) amplification.[24] Wild-type p53 also inhibits TYMS promoter activity, so mutated p53 represents a potential mechanism of target-associated resistance.[25] In aggregate, over 20 enzymatic targets comprise the 5-FU pharmacokinetic and pharmacodynamic pathway. Their true predictive value with respect to 5-FU response has yet to be thoroughly elucidated.

This review will summarize major targets with known involvement in the 5FU pharmacodynamic and pharmacokinetic pathway (Figure 1.2). Variation at either the gene, mRNA, or protein level for these targets is may potentially be associated with resistance to 5-FU. This chapter will review their biologic roles, their pharmacologic relevance with respect to 5-FU, available data on their involvement in 5-FU resistance, and their therapeutic utility as predictive markers for 5-FU efficacy. Based on this discussion, a targeted approach will be presented as a new tool for circumventing some of the technical challenges associated with traditional investigations of 5FU resistance.

Thymidylate Synthase

Thymidylate synthase (TS) is a critical enzyme for DNA synthesis. It catalyzes the methylation of 2V-deoxyuridine-5V-monophosphate (dUMP) to 2V-deoxythymidine-

5V-monophosphate (dTMP) thereby mediating the rate-limiting step of pyrimidine biosynthesis.[26] Accordingly, it is an important molecular target for many chemotherapy agents including 5-FU. Inhibition of TS by FdUMP, the active metabolite of 5-FU, is considered the primary mechanism of action of 5-FU. Numerous studies have evaluated polymorphisms and copy number aberrations in TS associated with variable response to 5-FU.Overexpression of TS has been linked to resistance to 5-FU. Notably, a polymorphic tandem repeat in the promoter region of the thymidylate synthase enhancer region influences TS expression and segregates 5-FU responders from non-responders.[27] Multiple studies show that three copies of the TS (TSER*3) tandem repeat gives greater TS expression than two copies (TSER*2).[28-30] Patients with stage 3 and 4 CRC who have TSER*2/TSER*2 or TSER*2/TSER*3 genotypes have improved response and survival from 5-FU compared to TSER*3 homozygous patients.[24,27]

TS levels have also been directly associated with 5-FU response in both advanced CRC and mCRC patients. Advanced CRC and mCRC patients with low TS levels have 2.2 and 3.3 greater odds, respectively, of responding to 5-FU compared to patients with high TS levels.[31] Additionally, data from multiple studies corroborate that tumors expressing high TS levels are associated with poorer survival than tumors expressing low TS levels.[32,33] Additionally, high thymidylate synthase gene copy number has been implicated in poor survival in mCRC patients receiving 5-FU.[34] Collectively, this data suggests that high TS levels, irrespective of cause (i.e. copy number, tandem repeat in enhancer region), are associated with poor response to 5-FU and shorter survival.

However, there are complicating factors that have limited the utility of TS as a biomarker including: the lack of standardized methodologies for assaying TS or TYMS and negative and even contradictory data. Additional studies are needed with consistent methodologies and 5-FU regimens that incorporate multiple TS variants and TS levels to delineate the true predictive value of TS. Non-TS variants also appear important in explaining 5-FU resistance and their integration into a model with TS would likely contribute to the overall predictive value.

Thymidine Phosphorylase

Thymidine phosphorylase (TP) catalyzes the reversible interconversion of thymine and thymidine, which are pyrimidine and nucleoside bases that are critical for DNA synthesis.[35,36] Additionally, since TP catalyzes a deoxyribosyl transfer reaction responsible for the biologic activation of 5-FU, it has also been investigated as a cause of 5-FU resistance in a variety of clinical settings.[21,37] However, the data is highly discordant. In CRC patients receiving 5-FU, tumor TP mRNA and protein levels have direct, no, or inverse associations with patient outcome, such as survival.[38-40] Presumably, low TP levels would be associated with poorer response or survival in patients receiving 5-FU since TP is responsible for activating the prodrug (5-FU). Nonetheless, in addition to bioactivation, TP also has dual and contradictory functionality as a proangiogenic factor.[41] Thus, the malignant phenotype of pronounced angiogenesis conferred by high TP may counterbalance or override any clinical utility of excess 5-FU activation. The predictive utility of TP levels alas remains convoluted.

Dihydropyrimidine Dehydrogenase

Dihydropyrimidine dehydrogenase (DPD) is an enzyme important for metabolic degradation of nucleotides, a process important for cellular detoxification, and the production of free pyrimidines that can be recycled for DNA and RNA synthesis. This activity is also important for 5-FU metabolism, as DPD mediates the initial and rate-limiting step of 5-FU catabolism.[42] DPD has been evaluated for its impact on 5-FU resistance. High DPD levels would be expected to correlate with poor 5-FU outcome since over 80% of 5-FU is catabolized by DPD.[43] However, similar to the TP data, the results are very ambiguous. Studies of CRC patients receiving 5-FU have shown direct, no, and inverse associations between tumor DPD mRNA and protein levels and 5-FU outcome.[44-50] Perhaps these discrepant results may be related to the reduced DPD expression and activity in colorectal tumors compared to normal mucosa.[51-54] The discordance may also be attributable to the non-standardized methods for measuring DPD levels. The lower DPD expression in some colorectal tumors may make a clear association between 5-FU resistance and DPD levels difficult.

Deoxyuridine Triphosphate

Deoxyuridine triphosphatase (DUT) is an enzyme that regulates intracellular 2V-deoxyuridine-5V-triphosphate (dUTP) levels. DUT catalyzes the conversion dUTP to dUMP, a critical substrate for TS. Thus, DUT is believed to indirectly modulate the effectiveness of 5-FU. Inhibition of TS by 5-FU results in accumulation of dUTP, which serves as a stimulus for uracil misincorporation into DNA and ultimately cellular death.[55] However, lack of accumulation of dUTP due to excess DUT would prevent uracil misincorporation into DNA and thereby result in 5-FU resistance.[55] Consequently, DUT

levels have also been investigated for 5-FU resistance. Ladner and colleagues found that response was exclusively seen in CRC patients who had low DUT ($p=0.005$).[55] Time to progression was also significantly longer ($p=0.017$) in patients with low DUT; however, there was no difference in overall survival ($p=0.09$). Knockdown of DUT with siRNA caused two of three cancer cell lines to become more sensitive to TS-inhibitor FUdR.[56] However, other *in vitro* studies indicated no significant association between altered cellular 5-FU resistance phenotype and DUT mRNA expression.[57] DUT appears to be an important target for 5-FU resistance, but its true predictive value has yet to be defined.

Uridine Cytidine Kinase 2

Uridine cytidine kinase (UCK) is an enzyme that catalyzes the phosphorylation of dUMP to deoxyuridine diphosphate (dUDP), the initial step in the production of the pyrimidine nucleoside triphosphates required for RNA and DNA synthesis. Since dUMP is the critical substrate for TS, it has been hypothesized that UCK ultimately affects 5-FU-mediated cytotoxicity.[58] Although UCK exists in two isoforms, UCK1 and UCK2, only UCK2 protein and mRNA levels correlate with UCK enzymatic activity.[58] Additionally, studies in rat pheochromocytoma cells and normal rat tissues suggest that the UCK pathway is a preferred pathway for 5-FU activation.[59] It was believed that overexpression of UCK2 would result in 5-FU sensitization; however, no difference in cytotoxicity existed in cells overexpressing UCK2 compared to cells with normal UCK2 levels.[59] Both colon tumor and CRC cell lines exhibited higher levels of UCK2 than normal tissue, other tumors, and other cell lines investigated.[58] The clinical relevance of UCK2 levels with respect to 5-FU activity is currently unknown, especially in lieu of the fact that 5-FU can be activated and mediate its cytotoxicity independent of UCK2.[60]

Ribonucleotide Reductase

Biologically, ribonucleotide reductase is an enzyme that produces deoxyoligonucleotides from ribonucleotides, a step that is essential for DNA synthesis and repair.[61,62] Human ribonucleotide reductase exists in two subunits RRM1 (regulatory) and RRM2 (catalytic).[61] Both subunits are necessary for enzymatic activity and are encoded by different genes on separate chromosomes. Since RRM1 and RRM2 are both critical for DNA synthesis, they have been evaluated by multiple studies as therapeutic targets in various cancers. Importantly ribonucleotide reductase reduces FUDP, a 5-FU intermediate metabolite, to FdUDP. FdUDP is subsequently dephosphorylated into FdUMP, the active 5-FU metabolite inhibiting TS mediating 5-FU's cytotoxicity. This pathway operates independent of TP and may be associated with 5-FU resistance. Recent data suggests that RRM1 and RRM2 may have conflicting roles in tumor malignancy. Whereas overexpression of RRM1 has significant inhibited tumor growth and incidence of metastases via PTEN (Phosphatase and Tensin Homolog), overexpression of RRM2 is associated with increased tumor invasiveness and metastases via various oncogenes. [63-67] Additionally, RRM2 protein level is significantly associated with the incidence of metastasis in colon cancer.[68] The pharmacologic significance of RRM1 and RRM2 with respect to 5-FU resistance in mCRC is presently unknown.

Non-metastatic cells

Non-metastatic cells (NME) is an enzyme encoded by the first discovered metastasis suppressor; presently there are over 20 known metastasis suppressor genes.[69,70] NME has many biological functions including: histidine kinase activity, binding of other proteins to regulate metastatic formation, and altering downstream gene expression. .[69,71]

Both NME1 and NME2 genes encode the hexamer nucleoside diphosphate kinase responsible for NME kinase function. Through phosphorylation, NME catalyzes conversion of UDP and dUDP to UTP and dUTP, respectively. Notably dUTP is a substrate for DUT and is ultimately used in DNA synthesis. NME's kinase activity is also responsible for activating 5-FU metabolites FUDP and FdUDP to FUTP and FdUTP, respectively, which is important for 5-FU cytotoxicity. Due to its biologic and pharmacologic roles, NME has also been investigated for its role in 5-FU resistance. Many studies have been conflicting: while some studies have found that NME1 expression (mRNA and protein) correlates with development of distant metastases, other studies have not. [72-76] Loss of heterozygosity (LOH) of the NME1 gene occurs in up to 52% of colorectal liver metastases.[77-79] However, some studies were unable to detect LOH in NME1.[80] It is unknown whether NME expression and LOH has predictive utility in mCRC patients treated with 5-FU.

Conclusions

5-Fluorouracil, a nucleotide analog, has successfully been used to treat a broad range of malignancies including: colorectal, head and neck, and pancreatic cancers. There are many pharmacogenomic targets within the 5-FU pharmacokinetic and pharmacodynamic pathway that are related to its response and resistance. However, the impact of their altered expression on 5-FU resistance in mCRC patients remains unknown. For example, TYMS (gene) amplification has been linked to 5-FU resistance in mCRC patients; however, data on the impact of altered protein expression of pathway targets in this patient group is unavailable, unconvincing or conflicting. Additionally, the impact of 5-FU exposure on the expression of the remainder of the 5-FU pathway

proteins remains unknown. Future studies are needed to investigate the expression of these targets in 5-FU sensitive and resistant mCRC patients to determine whether altered expression can explain 5-FU resistance.

Many studies unfortunately focus on single gene candidates. Despite gains in knowledge of pharmacokinetic and pharmacodynamic contributors to 5-FU resistance, the mechanistic determinants to 5-FU resistance remain unclear. With 24 proteins comprising the 5-FU pharmacokinetic and pharmacodynamic pathway known to date, it is quite improbable that 5-FU resistance would be wholly attributable to one target. Indeed, it is becoming apparent that the cause of 5-FU resistance is likely multifactorial.[21] Future studies must elucidate the complex interplay of pharmacokinetic and pharmacodynamic contributors to 5-FU resistance.

Another challenge in establishing clear associations between altered protein expression of 5-FU pathway genes and 5-FU resistance is that the qualitative means of detecting expression is subject to inter-evaluator variability. Qualitative methods of protein expression also suffer from lack of sensitivity, which can make finding associations between altered target expression and 5-FU resistance particularly difficult if the effect or sample size is small or modest. The nominal observations of traditional pathologist-based scoring of protein expression are unable to detect subtle differences in staining intensity. Thus, there is much disparity between the interpretations of observers with pathologist-based scoring. Additionally, traditional immunohistochemistry means of determining protein expression are slow. In future studies, researchers should avail

themselves to high-throughput, quantitative, sensitive, and unbiased tools for determining protein expression.

Introduction to Dissertation

5-FU is the cornerstone of treatment for colorectal and other gastrointestinal cancers, so its resistance represents a major clinical problem. Placing the 40-50% response rate of 5-FU based therapy into context means that not only are there 50-60% of patients who do not respond but also that the majority of those who do respond eventually relapse. Additionally, CRC accounts for approximately 10% of cancer-related deaths domestically. Colorectal cancer most commonly metastasizes to the liver; these metastases are surgically removed whenever feasible. Even with surgical and medical intervention, mCRC is disproportionately deadly with less than a 10% 5-year survival rate.[81] The focus of this dissertation project is to identify molecular determinants of 5-FU resistance in metastatic colorectal cancer. Consequently, this dissertation is separated into two sections. Part one centers on the protein expression results determined by automated quantitative analysis (AQUA) in mCRC patients sensitive and resistant to 5-FU. Part two is concerned with the mechanism for differential expression amongst 5-FU sensitive and resistant mCRC patients.

By carefully phenotyping patients who have consented to have their liver metastases banked at the UNC Tissue Procurement Facility, patients were stratified based on their 5-FU exposure. A comprehensive review of the clinical phenotyping database of mCRC patients and how it was used to explore 5-FU resistance is provided in Chapter 2. The purpose of Chapter 3 is to identify 5-FU pathway targets with altered expression

associated with 5-FU resistance in metastatic colorectal cancer patients using AQUA. Since protein expression will be quantified for multiple targets, this approach overcomes the aforementioned challenges of the single target-approach and the technical limitations of qualitative protein expression determination. Chapter 4 will communicate the mechanisms by which the 5-FU pathway targets associated with 5-FU exposure cause resistance. Lastly, the results of this research effort will be placed into context of the existing literature, and recommendations for a future research agenda that will advance the present results are provided in Chapter 5.

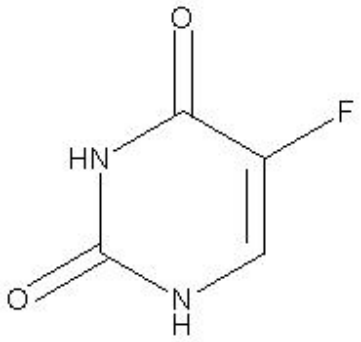

Figure 1.1 5-Fluorouracil Structure

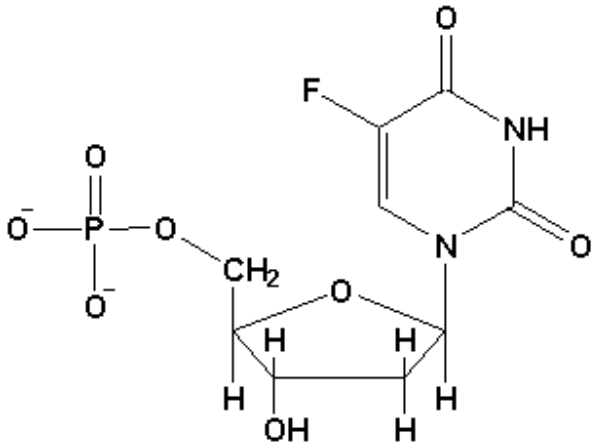

Figure 1.2 5-fluorodeoxyuridine Monophosphate (FdUMP) – the active metabolite of 5-fluorouracil.

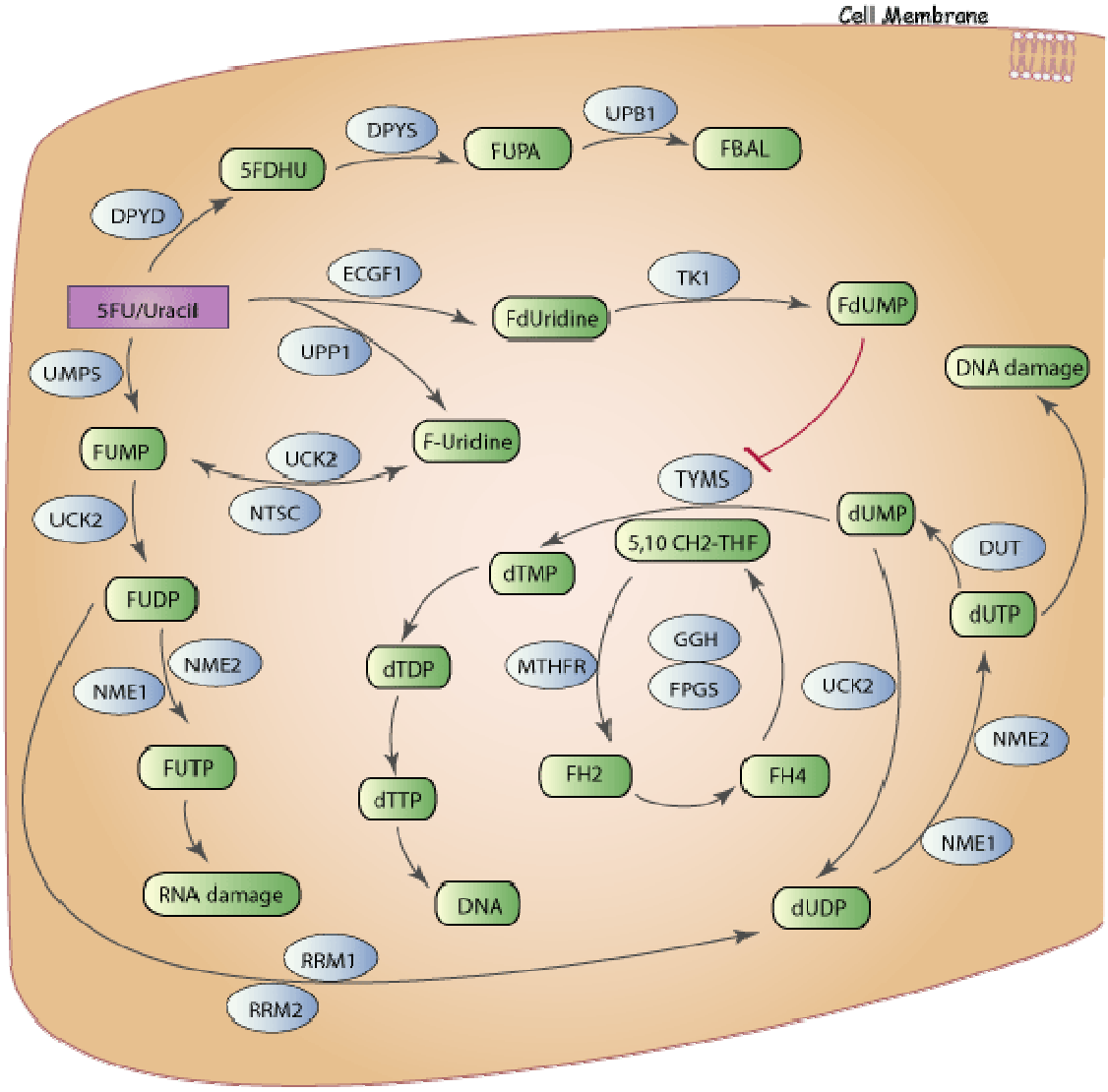

Figure 1.3 5-Fluorouracil Pharmacokinentic and Pharmacodynamic Pathway. T.E. Klein, J.T. Chang, M.K. Cho, K.L. et al."Integrating Genotype and Phenotype Information: An Overview of the PharmGKB Project" (220k PDF), *The Pharmacogenomics Journal* (2001) 1, 167-170.

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Chapter 2

Characterization of the Retrospective Clinical Cohort and Phenotyping Stratification

Abstract

Introduction: Colorectal cancer is the third most common non-cutaneous malignancy in the United States and the third most frequent cause of cancer-related deaths. The presence of distant metastases is particularly problematic, as the 5-year survival is 5-8%, despite 5-FU-based therapy. The primary objectives of this comprehensive review are to detail how data were collected to develop a clinical database resource that was used in subsequent studies investigating 5-FU resistance and to characterize the mCRC patients comprising this clinical cohort.

Methods: Electronic and paper patient records from North Carolina Memorial Hospital and community hospitals within the region were reviewed to obtain pertinent dates, chemotherapy regimens, treatment outcomes, and baseline disease characteristics. The Social Security Death Index was also queried to obtain survival data when unavailable from patient charts. Patients were only included if they had mCRC, had their colorectal liver metastases banked at North Carolina Memorial Hospital, and could be phenotyped with respect 5-FU exposure.

Results: Chart reviews for 140 patients were performed; one-hundred and twenty one of these patients had mCRC and could be phenotyped for 5-FU exposure. Sixty-seven of the 121 (55%) were exposed to 5-FU, and the remaining patients (54/121) were unexposed. There were no significant differences with respect to baseline characteristics with the exception of age at diagnosis of liver metastases ($p=0.01$). From surgical resection of colorectal liver metastases, the median overall survival was 885 days and median disease-free survival was 294 days.

Conclusions: Patients were well-characterized with respect to 5-FU exposure. The 5-FU exposed and unexposed groups did not significantly differ with respect to demographical data. This patient cohort is well-suited to investigate clinical resistance.

Introduction

Colorectal cancer (CRC) is the third most common non-cutaneous malignancy in the United States and the third most frequent cause of cancer-related deaths.[1] In 2010, an estimated 142,570 cases of CRC will be diagnosed and 51,370 people will die from this disease.[1] Despite the substantial progress that has been made in recent years, CRC remains a major clinical problem. The most critical prognostic factor for CRC is pathologic stage. The American Joint Committee on Cancer (AJCC) is the most common staging criteria used.[2,3] There is an inverse relationship between CRC patients' AJCC stage and their expected prognosis. As AJCC staging increases from stage 1 (early, localized primary tumor) to stage 4 (distant metastases), the 5-year survival decreases from 90% to less than 10%. Note that 30-40% of patients with localized CRC eventually develop distant metastases and nearly 20% of patients present initially with metastatic disease. Surgical resection of metastases in patients with a few metastases confined to a specific organ has been shown to be curative in some patients.[4] However, the majority of mCRC patients succumb to the disease.

Perhaps, the most insidious problem for mCRC patients is chemotherapy resistance, particularly 5-FU resistance since it is part of the therapeutic backbone of mCRC treatment. Although patients will initially respond to 5-FU based therapy, most develop recurrences. These patients will typically receive a new regimen that contains 5-FU, and while most won't respond, the few who do respond do so in an unpredictable manner. The lack of validated predictive markers for 5-FU response results in patients being exposed to unnecessary toxicity and a regimen with marginal chances of success. Often somatic changes within patient tumors are implicated as putative etiologies for 5-

FU resistance because they result in altered expression and copy number aberrations in 5-FU pathway proteins, mRNA, and genes. However, the results from clinical studies evaluating this resistance have been conflicting, and the studies are often underpowered and have limited external validity.[5-8]

It is critical that future studies not ignore the enriched phenotyping strategy. Clearly, the aforementioned data suggests that not all mCRC patients have the same response to 5-FU. The enriched strategy attributes differences in patient outcomes to underlying molecular differences. These molecular differences can be identified by comparing patients most likely to possess high concentrations of variation associated with resistance to those predisposed to be sensitive. An underlying presumption is the tumors remaining despite adequate 5-FU-based treatment are clinically-resistant to 5-FU. Giving such patients more 5-FU-based regimens would be expected to provide limited therapeutic utility while simultaneously exposing them to increased chemotherapy-related toxicity. Differences in gene copy number and expression are expected between 5-FU exposed and unexposed patients, as they represent two distinct mCRC patient populations: resistant and sensitive, respectively. Moreover, stratifying patients based on their 5-FU exposure capitalizes on the fact that exposed tumors are enriched with variation resulting in resistance that is not present in unexposed samples. When such variation has previously been identified through enrichment, it has been unambiguously implicated in 5-FU resistance.[9,10] As such, exposed tumors can be further exploited as a valuable discovery tool for investigating 5-FU resistance (Figure 2.1). Functional validation of these targets with knockdown studies would be expected to modulate the 5-FU resistance phenotype in CRC cells. By methodically characterizing mCRC patients

with respect to clinical resistance (5-FU exposure), these molecular etiologies underlying 5-FU resistance can be systematically dissected. Thus, while it is expected that exposed and unexposed patients will not significantly differ with regards to their baseline disease characteristics and demographics, the fundamental molecular heterogeneity between these two populations as a consequence of clinical resistance to 5-FU (exposure) serves as the basis for stratification and is the focus of this dissertation. Since confounding variables cannot be controlled for in this dataset, survival data will not be compared between exposed and unexposed. Accordingly, this chapter details how mCRC patients were phenotyped based on their 5-FU exposure and other pertinent clinical information and how these data were pooled to create the clinical database used for investigating 5-FU resistance in subsequent chapters.

Methods

Data Sources

Patient medical records were retrieved from colorectal cancer patients who had undergone surgical resection of their liver metastases at North Carolina Memorial Hospital between 1998 and February 2009. Paper and electronic charts from North Carolina Memorial Hospital and community hospitals within the region were reviewed. Survival data was also obtained from the Social Security Death Index when survival was unspecified in patient medical records. Examination of clinical records was approved by an institutional review board and was performed in adherence to Health Insurance Portability and Accountability Act regulations. A waiver of informed consent was granted.

Eligibility Criteria

Patients were included in this analysis if they met the following criteria: (1) had pathological diagnosis of metastatic colorectal cancer; (2) had colorectal liver metastases banked at the University of North Carolina Translational Pathology Laboratory (formally known as the Tissue Procurement Facility); (3) their exposure or lack of exposure to 5-FU for the immediate 6 months preceding resection of their liver metastases could be verified. Patients with ambiguous 5-FU exposure phenotype were excluded, such as patients where it is unclear whether they received 5-FU in the six months before their liver resection. All exposed patients received at least 4 weeks of 5-FU-based chemotherapy.

Data Extraction

The following data were extracted for each patient: first and last name, date of birth, gender, race, ethnicity, diagnosis data of primary and metastatic lesions, disease stage as determined per AJCC criteria, number and location of metastases, surgeon who performed resection of liver metastases, date of resection of liver metastases, radiofrequency ablation therapy, chemotherapy regimen within six months prior to resection of liver metastases, post surgery chemotherapy (regimen not documented), date of last follow up or death, date of recurrence or last pronouncement of no evidence of disease, height, weight, dosage reduction (when applicable), and referring physician. The data extraction was performed primarily by Roshawn Watson with some assistance from Michael Hudson and Joan Van Ord. Disagreements about data were resolved by consensus between the author, Christine Walko, and Bert O'Neil.

Statistical Analyses

All univariate statistical analyses were performed with SAS software, version 9 (SAS Corp, Cary, NC). $P<0.05$ was considered statistically significant. Overall survival was calculated from both a) the date of the surgical excision of the metastases and b) from the diagnosis date of metastatic colorectal cancer to the date of death or last follow-up. Survival was computed by the Kaplan–Meier method. Disease-free survival was determined from the date of the surgical excision of the metastasis to the date of recurrence or last declaration of no evidence of disease. Data were censored when patients were lost to follow-up.

Results

Demographics

The study population comprised of a total of 140 patients with colorectal liver metastases (Table 2.1). Nineteen patients eventually were excluded because they had inadequate documentation of presurgical chemotherapy, unconfirmed histology, noncolorectal primary tumors, or inadequate tissue available. The remaining 121 mCRC patients consisted of sixty-seven 5-FU exposed and fifty-four unexposed. Their ages at the diagnosis of their liver metastases ranged between thirty-eight and eighty-six (mean age of sixty-one). This cohort was comprised of sixty-one male patients and sixty female patients. The racial distribution of patients in this trial was: White, n = 90; Black, n =27; and Asian/Other, n=4. Other patient characteristics are listed in Table 2.1. The mean age at diagnosis in the unexposed group was 6 years greater than in the 5-FU exposed group (p=0.01); no other statistically significant differences in demographic data were observed between the two groups.

Survival

The median follow-up period of 434 days (95%confidence interval [CI], 335 to 533 days) for the 121 patients analyzed in this study. The median overall survival time was 885 days from surgical resection of liver metastases and 1308 days from diagnosis of liver metastases (Figures 2.2 and 2.3, respectively). The median disease-free survival time was 294 days from surgical resection of liver metastases (Figure 2.4).

Chemotherapy

Sixty-seven mCRC patients received 5-FU containing regimens within the six months preceding the resection of their liver metastases. The other components of the

regimens were also documented (see Table 2.1). Thirty-one (46%) patients received oxaliplatin as part of their regimen. Twenty-four (36%) patients received bevacizumab. Fourteen (21%) patients received irinotecan. One (1.4%) patient received cetuximab. Another patient was prescribed cetuximab but immediately discontinued usage after experiencing anaphylaxis during the administration of initial dose. Clearly, these chemotherapy regimens reflect the current diversity and complexity in the treatment of mCRC patients.

Discussion

Drug resistance is a primary reason for treatment failure and death in cancer patients. The causes of drug resistance in malignancies are relatively unknown, despite numerous investigations into the putative mechanisms. Recent data suggests that by comparing molecular differences between 5-FU exposed and unexposed patients, the underlying molecular contributors to 5-FU can be elucidated.[9] The presumptive hypothesis is that the residual tumor post-5-FU exposure is enriched with somatic changes causing the resistance whereas the clinically-sensitive tumors would respond to an adequate course of 5-FU. A relevant *in vivo* example is the genetic alterations that occur in the BCR/ABL gene in chronic myelogenous leukemia patients during Gleevac treatment. These alterations unequivocally suggest that the BCR/ABL gene plays a significant role in Gleevac resistance.[11,12] Similarly, changes in 5-FU targets that are only evident tumors already clinically resistant (residual tumor) to the 5-FU are unambiguously associated with 5-FU resistance.

Using this model as the basis investigate drug resistance, a clinical cohort was developed consisting of both 5-FU exposed and unexposed patients. The results demonstrate that the exposed and unexposed patients do not significantly differ in baseline disease characteristics and demographics with one minor exception: the mean age of the unexposed patients was 6 years greater than exposed patients. The implications are that differences between the exposed and unexposed will be attributable to 5-FU exposure, clinical resistance to 5-FU, and will have relevance to the clinical management of mCRC.

A strength of this design is that it allows for clinical resistance to be evaluated using *in vivo* samples from well-characterized patients. Additionally, the cohort consists of patients from both community and university settings, which means data from this cohort reflects real-world 5-FU resistance in mCRC patients. The fact that all included patients had resectable disease is also notable. Historically, only 10-20% of patients with colorectal liver metastases are resection candidates, and this cohort is among the largest studies investigating 5-FU resistance in this patient cohort.

There are also some important considerations when evaluating data from this cohort. First, by excluding non-resection candidates, the study design screens out patients with the most aggressive disease. Resection candidates typically have higher performance status and more contained liver disease than non-resection candidates. In contrast to the overall 5-year survival of 5-8% seen in mCRC patients, survival in patients amendable to resection is significantly higher at 40-50%.[13-17] Nonetheless, resection candidates have significant resistance considering that they are still prone to numerous recurrences and only 50-60% survive at 5 years. Second, chemotherapy prior to six months before resection is not documented, so some patients in unexposed group likely received 5-FU before this point. There was no precedent dictating when data collection started, so by consensus of medical oncologist and gastroenterologists, six months preceding resections was agreed to be reasonable. Third, patients who were exposed to 5-FU were often treated with other agents, so their influence cannot be separated from 5-FU. The patient cohort examined in the Wang et al study, were treated with only 5-FU, as this was the standard of care therapy at that time in history.[9] However, the treatment guidelines have since evolved. The addition of concomitant agents has been shown to improve response

and survival, so 5-FU monotherapy is no longer gold standard. This is a common consideration in any contemporary *in vivo* study investigating 5-FU resistance and certainly not limited to this analysis.

It is tempting to directly contrast survival of the unexposed and exposed patients; however, there are several limitations to the validity of such a comparison. First, survival would be influenced by post-surgical access to care and patient preferences. Since both of these were not assessed, they would be important potential confounders to a survival analysis. Second, the drugs included in the post-surgical adjuvant therapy, duration of therapy, and additional procedures were also not included in the database, yet they can all influence survival. Lastly, the presence of extrahepatic disease in mCRC patients has repeatedly been shown to decrease survival, so a meaningful survival analysis should also be adjusted to reflect these data.[18-23] Acquisition of the aforementioned data was beyond the scope and resources for this project. Thus, a comparison of survival based on 5-FU phenotype, without controlling for these confounders, would make interpretation ambiguous and was never planned.

The primary conclusions drawn from this review are that the exposed and unexposed patient groups do not significantly differ with respect to baseline disease characteristics and demographics. Also, the included patients are a distinct subset of mCRC patients who were eligible for resection. In aggregate, the data collected on this clinical cohort will be critical to determining molecular contributors to 5-FU resistance in mCRC patients. Based on the available data and samples, the database has lent itself to numerous types of analyses including expression analysis (Chapters 3 and 4), copy

number analysis (Chapter 4). Overall, stratifying patients on the basis of 5-FU exposure is a pragmatic and proven study design for investigating clinical resistance to 5-FU.

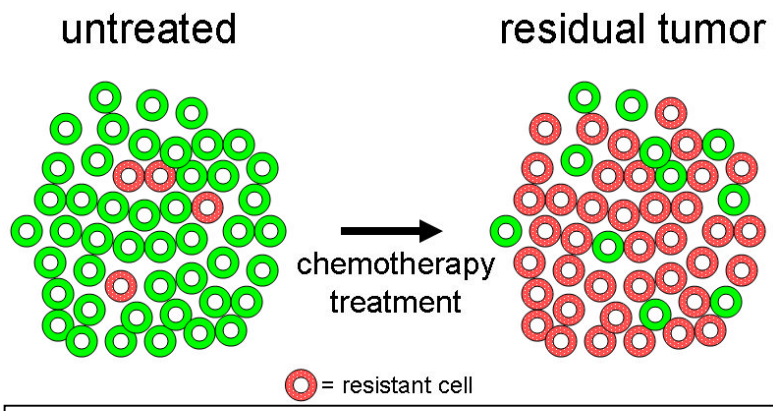

Figure 2.1 Model for an 'enriched' approach for genomic discovery. The hypothesis is that residual tumor after chemotherapy treatment is clinically resistant and thereby harbors functionally important somatic alterations.

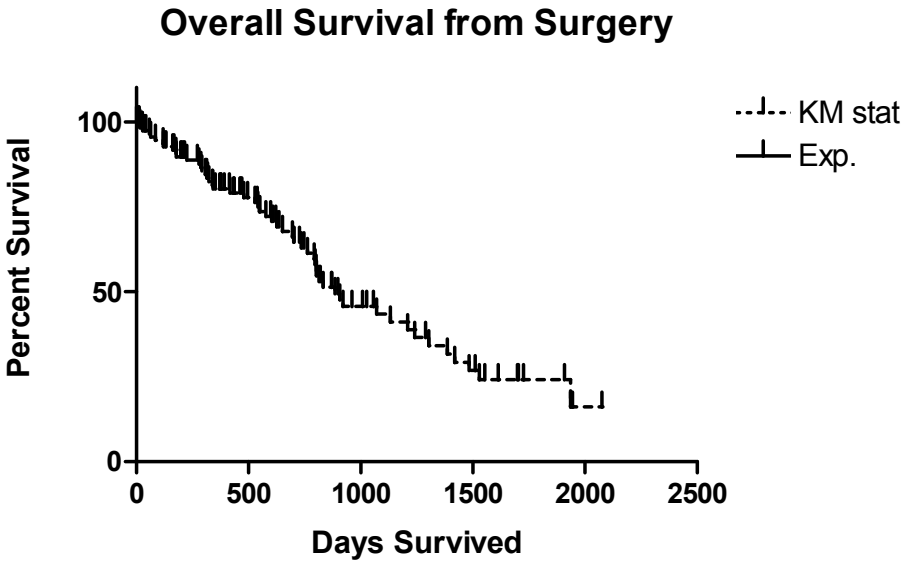

Figure 2.2 Kaplan-Meier curve depicting the overall survival of the entire clinical cohort from surgery.

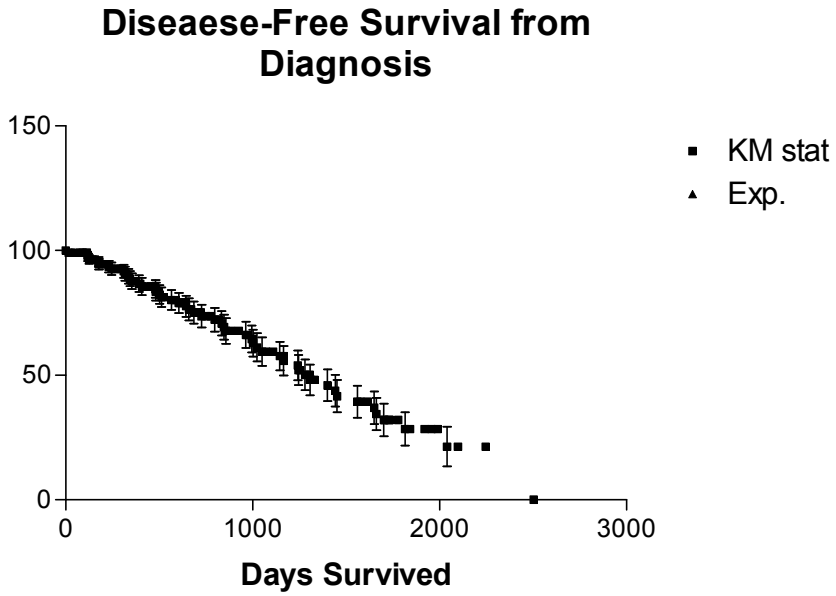

Figure 2.3 Kaplan-Meier curve depicting the disease-free survival of the entire clinical cohort from diagnosis of liver metastases.

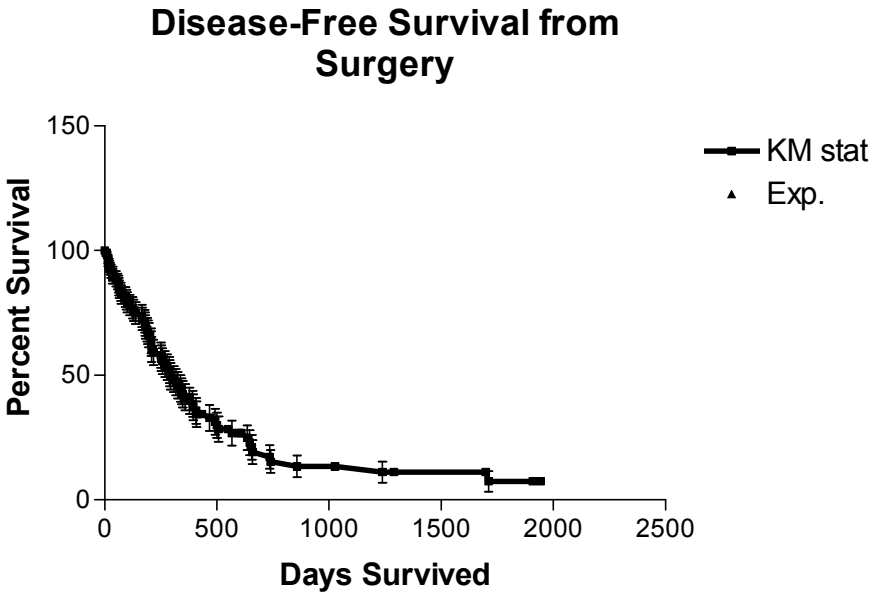

Figure 2.4 Kaplan-Meier curve depicting the disease-free survival of the entire clinical cohort from surgical resection of liver metastases.

Characteristic	Unexposed	5-FU Exposed	P
Gender			
Male	31 (57%)	30 (45%)	NS
Female	23 (43%)	37 (55%)	
Race/Ethnicity			
White	38 (70%)	52 (78%)	
Black	13 (24%)	14 (21%)	
Asian/Other	3 (6%)	1 (2%)	
Hispanic	0	1 (2%)	NS
Age (mean) at diagnosis[1]	65 (range 42 – 86)	59 (range 38– 85)	0.01
Tumor			
1	5	2	
2	8	5	
3	22	42	
4	3	2	NS
Nodes			
0	28	26	
1	15	19	
2	9	16	NS
Metastases[2]			
0	32	29	
1	18	35	NS
Initial No. of Metastases			
1	28	26	
2	11	12	
>2	7	15	
Range	1-6	1-14	
Median	1	1.5	NS
Neoadjuvant chemotherapy (%)[3]			
5-Flourouracil-containing regimen		67 (100)	
5-Fluorouracil-Oxaliplatin regimen		31 (46)	
5-Fluorouracil-Irinotecan regimen		14 (21)	
5-Fluorouracil-Bevacizumab regimen		24 (36)	

[1]Diagnosis refers to diagnosis of liver metastasis
[2] TNM criteria was taken at diagnosis of colorectal cancer not diagnosis of liver metastases
[3]Regimens included more than one agent

Table 2.1 Clinical cohort demographics and baseline disease characteristics.

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Chapter 3

Quantitative Determination of Expression of 5-FU Pathway Proteins and Functional Validation

Abstract

Introduction: Multiple proteins within the 5-fluorouracil (5-FU) pharmacokinetic and pharmacodynamic pathway have been implicated in 5-FU resistance. Given that 5-FU is the mainstay of chemotherapy for colorectal cancer, its resistance represents a major clinical problem. In light of the high mortality associated with colorectal cancer, novel alterations in protein expression were sought to determine which targets were associated with clinical 5-FU resistance in vivo. Overexpression of UCK2, DUT, and DPD, 5-FU pathway proteins, have all been linked to clinical resistance in gastrointestinal cancers. The primary objective was to identify 5-FU pathway proteins with altered expression that is significantly associated with clinical resistance to 5-FU.

Methods: Automated quantitative analysis (AQUA), a fluorescence based method for analysis of in situ protein expression, was used to determine expression of 5-FU pathway proteins, specifically DPD, DUT-N, NME1, RRM1, RRM2, TS, TP, and UCK2 in a cohort of colorectal cancer patients. The patient cohort consisted of 47 5-FU exposed and 41 5-FU unexposed patients.

Results: Of the eight proteins, univariate analysis shows that UCK2 had higher protein expression in liver metastases from 5-FU exposed colorectal cancer patients than from 5-FU unexposed patients ($p=0.007$). Additionally, the multivariate analyses for DUT ($p=0.0135$), DPD ($p=0.035$), and UCK2 (0.019) showed that 5-FU exposed patients had higher expression of these protein targets compared to the unexposed.

Discussion: These results indicate that UCK2, DUT, and DPD expression differs in colorectal liver metastases that are clinically resistant to 5-FU compared to unexposed samples. These novel findings have important implications to the selection of appropriate therapy for patients treated with 5-FU.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in the U.S., accounting for an estimated 142,570 cases new diagnoses in 2010.[1] Approximately, 20% of the newly diagnosed patients already have metastatic colorectal cancer (mCRC), which has a dismal 5-8% overall survival.[1] 5-FU is most active agent against colorectal cancer and is the most commonly used chemotherapeutic in gastrointestinal cancers. Other anti-neoplastic drugs are added to increase 5-FU efficacy, but response rates typically do not exceed 50% for both advanced CRC and mCRC.[2-4] Additionally, many of the initial responders relapse, as recurrences occur in 27-38% of stage 2 and 3 patients and nearly all mCRC patients by 5-years.[2,4-8] Second- or third-line chemotherapy is typically even less effective and can be associated with substantial toxicities. This certainly underscores the importance of identifying the molecular predictors of 5-FU efficacy as nearly all patients will receive 5-FU.

Proteins critical to 5-FU pharmacokinetics and pharmacodynamics are involved in the de novo synthetic and salvage pathways that generate nucleotides, the precursors to nucleic acids. The levels of these pathway proteins must be accurately regulated and coordinated to prevent disruption of cellular metabolism. Defects in synthesis, regulation, and recognition of growth factors can result in carcinogenesis. As a nucleoside analog, 5-FU exploits this pathway primarily through its inhibition of TYMS, a key pathway enzyme responsible for pyrimidine synthesis. Incidentally, the bioactivation and catabolism of 5-FU is also dependent on proteins within this pathway, so the levels of key pathway proteins may accordingly modulate tumor sensitivity to 5-FU. However,

previous investigations have been variable with positive, negative and no associations being found with the levels of pathway targets and resistance, so there is no clear consensus for the molecular etiology for 5-FU resistance.[9-11] Such disparate results might be explained by the fact that all of these earlier reports relied on manual quantification of protein levels by conventional immunohistochemistry methods, which suffer from limited sensitivity, inter-evaluator biases, and lack of reproducibility.

Manual quantification is limited by pathologists' abilities to accurately and precisely score tissues by eye, often using categorical scoring that can differ significantly between experimenters. Additionally, the heterogeneous nature of tumors complicates the analysis of histological samples.

One histological tool that is revolutionizing how samples are being analyzed is the tissue microarray. Tissue microarrays (TMAs) consists of paraffin blocks holding up to 1000 individual tissue cores assembled in an array to allow for high-throughput, multiplex histological analysis. There are numerous benefits of using TMAs. TMAs allow for all specimens to be analyzed simultaneously thereby reducing inter-sample variability associated with technical difference between assays. An additional advantage of using TMAs is that they can be paired with highly sensitive quantitative analytical tools thereby allowing greater discrimination between small differences in the expression levels of targets can be discerned over the aforementioned semi-quantitative methods. Another major benefit is that quantitative analysis allows for continuous scoring of target expression rather than a categorical proxy. This is very important in determining the predictive value of a marker's expression because human variability in scoring

immunohistochemical staining by eye can diminish the reproducibility of assessment. Also, many targets have variable expression, so validating the predictive utility of the expression of markers necessitates consistent criterion being applied.

Automated quantitative analysis (AQUA) is a set of algorithms that uses immunofluororescence to rapidly and reproducibly quantify protein expression of specimens arranged in a TMA. AQUA quantification of a protein of interest will typically occur within user-defined subcellular compartments within the tumor region for each tissue microarray spot.[12] AQUA has previously been validated and used to determine the prognostic relevance of thymidylate synthase (TS) expression in two cohorts of colorectal cancer patients.[13] The 5-year disease free survival was significantly decreased in patients whose tumors had high nuclear to cytoplasmic TS ratios ($P<0.03$).[13]

Wang et al. demonstrated that distinct differences exist between mCRC patients exposed to 5-FU compared to those who are unexposed, and these differences were associated with 5-FU resistance.[14] Patients were stratified based on whether they received 5-FU and were assessed for thymidylate synthase gene copy number. Thymidylate synthase amplification only occurred in patients exposed to 5-FU, and patients without thymidylate synthase amplification had a median overall survival 4 times longer than the patients with TYMS amplification (5-FU exposed; $p=0.007$). Thus, thymidylate synthase amplification was significantly associated with 5-FU resistance.

To date, only TYMS expression levels have been assessed by AQUA to gain insight into resistance mechanisms in colorectal patients treated with 5-FU. Manual quantification of other proteins known to be involved in the 5-FU pharmacokinetic and

pharmacodynamic pathway have met with conflicting results. A quantitative evaluation of these proteins may lend insight into mechanisms of resistance for this important drug. This paper explores the hypothesis that 5-FU exposed and unexposed colorectal liver metastases have molecular differences in 5-FU pathway protein levels that are associated with 5-FU resistance. Using AQUA, the expression of eight 5-FU pathway proteins will be quantified *in situ* to delineate which are associated clinical resistance to 5-FU.

Methods

Tissue microarray construction and cohort details

A tissue microarray was constructed from archival formalin-fixed, paraffin-embedded colorectal liver metastases. H&E-stained full sections were used to determine representative regions to core. The tissue microarray was constructed with three 0.6-mm-diameter cores of each case spaced 0.8 mm apart in a grid format using a TMArrayer (Pathological Devices, Westminster, MD). The tissue microarray block was then cut into 4 micron sections with a microtome. The colorectal cancer cohort consists of 88 samples of colorectal liver metastases from the University of North Carolina Anatomical Pathology Laboratory archived between 1998 to 2008. The cohort contains samples from forty-seven 5-FU exposed patients and forty-one 5-FU unexposed patients. The mean and median follow-up time for the entire cohort is 485 days and 383 days, respectively. The mean age of diagnosis of liver metastases is 58 years for patients exposed to 5-FU and 64 for unexposed patients. Chemotherapy regimens for the 6 months preceding resection of colorectal liver metastases were documented, and patients were stratified based on their exposure to 5-FU during this period. All exposed patients were treated with at least 4 weeks of 5-FU within the six months prior to resection.

Cell line culture and harvest

The following cell lines were used in this study: SW-48, SW-620, WiDr, HT-29, HCT-116, Lovo, and SW-480 which were donated by other labs. HCT-116, Lovo, SW-48, and SW-620 were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). SW-480, HT-29, and WiDr were cultured in DMEM with 10% FBS. All cell lines were

maintained in at 37°C with 5% CO_2. The cell lines were cultured on 75cm^2 tissue culture flasks. After cells reached 80% confluence, they were washed twice in 1x PBS and 5 mL of neutral buffered formalin added at room temperature. The cells were then removed from the flask and refrigerated (4°C) in neutral buffered formalin. The cells were centrifuged at 1,500 rpm at room temperature for 10 minutes. The supernatant was then removed, and the cells were resuspended in 80% ethanol. The cells were centrifuge for 5 minutes at 12,000 rpm, so that supernatant can be removed. The remaining cell pellet was then resuspended in 50-100 uL of warm low melt agarose, and allowed to solidify. The cell block was then placed in a tissue cassette, processed, and paraffin embedded. This served as the donor block. A donor block of each cell line was created so that all of the cell lines could be assembled on a single cell line array in triplicate.

Immunofluorosecent staining

The tissue microarray and cell line microarray slides were heated at 60°C for 30 minutes. The slides were then deparafinized with 3 xylene rinses for 5 minutes each. Next, the slides were rehydrated with two 100% ethanol, 95% ethanol, 80% ethanol rinses 70% ethanol rinses for two minutes each. Afterwards, the slides were rinsed with Wash Buffer 1X (Dako, Carpinteria, CA). Antigen retrieval was performed by boiling the slides in Antigen Retrieval solution (Dako) in a pressure cooker for 20 minutes and then letting it cool down to room temperature. The slides were rinsed again briefly with Wash Buffer 1X (Dako) and then incubated with peroxidase block (Dako) for 30 minutes. Next slides were incubated with protein block (Dako) for another 20 minutes. Slides were then rinsed in Wash Buffer 1X (Dako). Next, samples were incubated with the primary antibodies. Slides were hybridized to one of following primary antibodies: TS

(thymidylate synthase, 1:250 dilution for 1 hour, Abnova), TP (thymidine phosphorylase, 1:266 dilution for 1 hour, Abcam), DPD (dipyrimidine dehydrogenase, 1:46 dilution for 1 hour, Abnova), DUT-N (deoxyuridine triphosphate-nuclear, 1:2000 dilution for 1 hour, Abcam), NME1 (non-metastatic cells-1, 1:266 dilution for 1 hour, Novus), RRM2 (ribonucleotide reductase-2, 1:250 dilution for 1 hour, Abcam), UCK2 (uridine-cytidine kinase 2, 1:50 dilution for overnight at 4°C, Abcam), and RRM1 (ribonucleotide reductase-1, 1:100 for overnight at 4°C, Millipore). Concurrently with primary antibody incubation, slides were stained with either pancytokeratin mouse (for TS, TP, DUT-N, RRM1, RRM2, NME1, DPD, at 1:200 dilution) slides or a rabbit anticytokeratin antibody (for UCK2, at 1:400 dilution, clone EPR1622Y, Epitomics). Afterwards, slides were incubated with secondary antibodies for 1 hour at room temperature; Alexa 555 goat anti-mouse and Alexa 555 goat anti-rabbit antibody (Invitrogen) 1:200 dilution were used to stain the cytokeratins and species-specific horse radish peroxidase (Dako). Slides were washed with wash buffer (Dako) and then incubated with Cy-5 tyramide (1:50 dilution in Amplification Diluent, Perkin-Elmer) for 10 minutes. Slides were mounted in Prolong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI, 1:100, Invitrogen) for imaging the nuclei and coverslipped.

AQUA analysis of microarrays

AQUA is paired with fluorescence microscopy to generate images of the microarrays that can be used to quantify the proteins of interest within the tumor of each core, according to previously published methods.[12,13] Briefly, pan-cytokeratin is used to differentiate epithelial tumor from stroma and fluorescent tags (i.e. DAPI). Pan-cytokeratin also enables subcellular localization of targets (proteins of interest) and nuclei

(i.e. DAPI). For instance, areas within the tumor mask, labeled with pan-cytokeratin (Cy3, green), lacking DAPI were considered ‘‘nonnuclear’’ or ‘‘cytoplasmic.’’ The target markers (DPD, DUT-N, NME1, RRM1, RRM2, TS, TP, and UCK2) are visualized with Cy5 (red). Images were obtained of each histospot, and the corresponding AQUA score represents the abundance of a protein of interest by pixel intensity. Tissue microarray cores lacking sufficient colon tumor epithelium (<5% of total area has pan-cytokeratin mask) were automatically excluded from this study. This exclusion decreased the total number of samples evaluated for each of the biomarkers to between 221 and 243

Plasmid shRNA, Transfection, and Cytotoxicity

The shRNA gene knockdown work was performed as previously reported.[15] 5-FU pathway targets were validated with shRNA-mediated gene knowdown in three colorectal cancer cell lines: HCT-116, HT29, and SW620. The OpenBiosystems plasmid shRNA bacterial glycerol stocks for each protein were obtained through the University of North Carolina at Chapel Hill Lenti-shRNA Core. Plasmid isolation was conducted using the Macherey-Nagel (MN) NucleoSpin Robot-96 plasmid kit (Bethlehem, PA, USA) according to the manufacturer’s protocol. Bacteria were inoculated in Terrific Broth medium (MP Biomedicals, Solon, OH, USA) containing 100µg/ml of Carbenicillin (Invitrogen, Carlsbad, CA, USA) on 96-well MN Square-well Blocks, and grown in a GeneMachine HiGro rotative incubator (San Carlos, CA, USA).

Exponentially growing (passages 5-15) colorectal cancer cell lines (HCT-116, HT29, and SW620) were seeded at a density of 20,000 cells per well in 96 well plates and incubated overnight. The following day, media was removed by aspiration and

replaced with 50µl of warm fresh complete media. Cells were transfected with plasmid-liposome complexes composed of 100ng plasmid shRNA and 0.5 µg Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a final volume of 50 µl OPTIMEM I (Invitrogen, Carlsbad, CA, USA). The following day, transfection media was removed by aspiration, and cells were dissociated after incubation with 0.05% trypsin-53 mM EDTA in HBSS (Cellgro, Manassas, VA, USA). Transfectants were seeded in 384 well plates at a density of 1,000 cells/well and plates were incubated at at 37°C with 5% CO_2 for an additional 24 hours.

These transected cell lines were then exposed to increasing concentrations of 5-fluorouracil ranging from 0.001 to 1000µM. After 96 hours of drug exposure, the vital dye indicator, alamarBlue (10% final concentration, Invitrogen Carlsbad, CA, USA) was added. Fluorescence was measured at Ex 535 and Em 595 using a Tecan 1000 Multiplate Reader (Männedorf, Switzerland) 6-8 hours following alamar blue addition. The fluorescence readings were corrected for the blank (cells exposed to vehicle alone), before being converted into percent cell survival at each drug concentration, and the IC_{50} determined utilizing XLfit4.0 software from IDBS (Guildford, UK).

Statistical Analysis

Comparisons of baseline demographics and disease characteristics between exposed and unexposed samples were performed using the Fisher's exact test. The differences expression of each target between the exposed and unexposed samples were evaluated using the Wilcoxon Rank Sum test. Logistic Regression was used to evaluate associations of AQUA scores, adjusted for clinicopathologic variables, with group status.

All analyses were done using SAS v9.2 (SAS Institute, Inc, Cary, NC). A plasmid shRNA was considered as "active" when the IC_{50} was outside the range mean at the control vector eGFP IC_{50}±3SD. In a given cell line, a gene with two or more active plasmids producing the same cytotoxicity phenotype was considered as "active gene". A gene was selected as "credentialed" in the 5-FU pathway when its knockdown significantly altered IC_{50s} in the HCT-116 cells (p53 wt) in addition to at least one of the two cell lines with mutated p53 (SW620 or HT-29 cells).

Results

Validation

To reliably measure the levels of the 5-FU pathway targets, total protein levels of DPD, DUT-N, and NME1 were determined independently by Western blot and by AQUA in cell line controls (Figure 3.1). Densitometry quantification of Western blot bands was performed, and the 5-FU pathway targets were normalized to the β-tubulin control to determine their relative expression. The correlations between Western Blot and AQUA were strong overall. Pearsons correlation for the two methods ranged between 77-91% and were significant for DPD and DUT-N (p=0.0042 and p=0.045). The correlation for NME1 was trending towards significance (p=0.075). The intratumoral heterogeneity among redundant tumor cores was high (Linear Regression R^2<0.3, P=N.S. all targets), so median AQUA scores of redundant histospots were used.

Demographics

Samples were annotated with demographic, clinical, and follow-up information (Table 3.1). For the purpose of this study, the cohort consists of 88 metastatic colorectal cancer patients (47 5-FU exposed and 41 unexposed). Their ages from the time of diagnosis of liver metastases ranged between 41 and 85 (mean age of 61). This cohort consisted of forty-three male patients and forty-five female patients. The racial distribution of patients in this trial was: White, n = 65; Black, n =20; and Asian/Other, n=3. Other patient characteristics are listed in Table 3.1. The mean age at diagnosis in the unexposed group was 6 years greater than in the 5-FU exposed group (p=0.01); no

other statistically significant differences in demographic data were observed between the two groups.

Univariate analyses

The association of 5-FU exposure with the expression of 5-FU pathway targets was assessed for all patients. Representative AQUA images are shown in Figure 3.2. The results demonstrate that the colorectal liver metastases have variable expression of 5-FU pathway proteins. Exposed tumors had a significantly higher median UCK2 expression than unexposed tumors (AQUA scores 8152.50 versus 6706.42, respectively, $p=0.007$). There were no significant differences between the median expression of the exposed and unexposed tumors for the other targets although exposed patients were trending towards higher DUT-N expression, $p< 0.058$.

Multivariate analysis

To determine whether clinicopathological parameters, target expression, and the nuclear to cyptoplasmic ratio were significantly associated with 5-FU exposure and pathway proteins, a multivariate logistic regression analysis was performed on the cohort. Specifically, age, gender, target protein expression in tumor mask, tumor size, nodal status, and nuclear to cytoplasmic protein expression ratio (as both a categorical and continuous variable) were added into the model. Cases with missing values were excluded from the analysis (Tables 3.2, 3.3, and 3.4). The best model comprised of gender, race, and protein expression level in 1000 unit increments. DPD protein expression is significantly higher in exposed samples (Point estimate 1.62; 95% confidence interval [95% CI], 1.03-2.53; $p=0.035$), when the model adjusts for gender

(Point estimate 3.34; 95% CI, 1.28-8.68; p=0.0135) and race (Point estimate 0.61, 95% CI, 0.21-1.72; p=0.347). DUT protein expression was also significantly higher in exposed samples (Point estimate 1.32; 95% confidence interval [95% CI], 1.05-1.66; p= 0.019), when the model adjusts for gender (Point estimate 3.29; 95% CI, 1.28-8.46; p=0.013) and race (Point estimate 0.62, 95% CI, 0.22-1.76; p=0. 367). The exposed samples also had higher UCK2 expression than unexposed samples (Point estimate 1.28; 95% CI, 1.06-1.54; 0.01) when the model corrected for gender (Point estimate 2.83; 95% CI, 1.11-7.22; p=0.029) and race (Point estimate 0.50; 95% CI, 0.17-1.46; p=0.206). Expression of the other protein targets did not significantly differ between exposed and unexposed samples using the multivariate model. Nuclear to cytoplasmic protein expression ratio (as a categorical and continuous variable), tumor stage, and nodal status did not significantly interact with 5-FU exposure phenotype, race, or gender, so they were not included in the final model because of parsimony.

Functional Analysis

To assess the functional relevance of the three proteins (DPD, UCK2, and DUT) associated with clinical resistance to 5-FU using AQUA analysis, shRNA gene knockdown was performed in three colorectal cancer cell lines. Knockdown of UCK2 resulted in a significant decrease of the IC_{50} by a magnitude of 3- to 6-fold in all three cell lines. DUT knockdown also produced a significant decrease in 5-FU IC_{50} following knockdown, causing a 13- and 3-fold reduction in HCT-116 and SW620 cells, respectively. The knockdown of DPYD, the gene encoding DPD, significantly decreased the 5-FU IC_{50} of SW620 by a magnitude of 6-fold; its knockdown also resulted in nearly

a 2-fold decline in the 5-FU IC_{50} in the HCT-116 and HT-29 cell lines but was just below the threshold of significance.

Discussion

Patients with mCRC have variable responses to 5-FU even with identical treatment and clinicopathologic parameters. This inconsistency is perhaps attributable to molecular differences modulating disease pathology and cancer resistance to 5-FU. Recent studies have demonstrated differences in biomarker levels are associated with chemotherapy response in other malignancies. For example, the human epidermal growth factor receptor 2 (HER2) is amplified or overexpressed in 20-25% of breast cancers.[16-18] Breast cancer patients with HER2 positive (HER2+) tumors have a worse prognosis in absence of therapy, and response to trastuzumab has almost exclusively limited to breast cancer patients with HER2 amplification as determined by FISH.[19-24] Despite numerous investigations, underlying determinants of 5-FU resistance in colorectal cancer remain elusive. Since 5-FU is the therapeutic backbone for treatment of advanced and metastatic colorectal cancer, determining molecular contributors to its resistance would have a meaningful impact on therapy.[25,26]

The expression of many 5-FU pathway proteins has not been studied in colorectal metastases, and the expression data for those that have been investigated are conflicting. For example, expression levels of the pathway target TS have been reported to have positive, negative and no association with 5-FU resistance. Liver metastases expressing high levels of TS protein have been associated with lack of clinical response to 5-FU in vivo.[27] Data also suggest that tumors expressing high TS levels are associated with poorer survival than tumors expressing low TS levels.[28,29] However, other studies show that patients treated with adjuvant chemotherapy with low TS protein have impaired survival

outcome compared to their high TS expressing counterparts. [30,31] Similar expression studies were performed for thymidine phosphorylase, TP, but data has not consistently demonstrated that overexpression is linked to good or poor clinical response.[32] Conflicting data also exists linking DPD protein expression to 5-FU resistance. Studies of CRC patients receiving 5-FU have shown both direct, no, and inverse associations between tumor DPD mRNA and protein levels and 5-FU outcome.[10,33,34] The general consensus is that the role of tumoral DPD levels as a prognostic factor for clinical responsiveness has not been firmly established.[35] Due to the limited and ambiguous *in vivo* data on the relationship between the expression of the 5-FU pathway proteins and 5-FU resistance, further investigations into the molecular profiles of 5-FU pathway proteins in clinically resistant and sensitive colorectal cancer samples may provide information pertinent to patient therapy. Characterization of differential expression associated with 5-FU exposure may be critical to identifying the molecular determinants of 5-FU resistance.

The expression of eight important 5-FU pathway proteins was evaluated in 88 colorectal liver metastases stratified by 5-FU exposure. Three of these 8 proteins had significant differences in expression between exposed and unexposed tumors in the multivariate analysis (UCK2, DPD, and DUT). Differential UCK2 expression between exposed and unexposed samples was also seen in the univariate analysis, and DUT expression was just below the threshold for significance. The significant differences in expression supports the presence of distinct molecular phenotypes associated with clinical resistance to 5-FU. Due to DPD, DUT, and UCK2 prominent biological roles in nucleic acid synthesis, their involvement in 5-FU resistance is not surprising. As a

nucleoside analog, 5-FU mediates its cytotoxicity via targeting the pyrimidine biosynthetic pathway.

DPD is responsible for the first and rate-limiting step of 5-FU catabolism and mediates over 80% of 5-FU elimination. Therefore, higher levels of DPD, as seen in the exposed samples, would be expected to be associated with 5-FU resistance presumably because more of the drug would be catabolized. Studies have shown both no and an inverse association between tumor DPD protein levels and the magnitude of 5-FU sensitivity via multiple methods.[10,33,34] The lack of correlation is at least partly imputable to non-standardized method for measuring DPD levels. Rat *in vitro* studies suggest that the UCK pathway is a preferred pathway for 5-FU activation.[36] Consequently, an abundance of UCK would presumably cause sensitization to 5-FU; however, cells overexpressing UCK2 had no difference in 5-FU cytotoxicity compared to cells expressing normal UCK2 levels.[36] Additionally, DUT is a critical enzyme in the 5-FU pathway because produces an essential substrate for TS. It is reasonable that DUT levels would be associated with 5-FU resistance. Previous worked has linked low DUT expression with both response to 5-FU ($p=0.005$) and a longer time to progression ($p=0.017$) but not overall survival ($p=0.09$).[37] While the true predictive value of DUT levels has remains to be clearly delineated, it does appear to be an important target for 5-FU resistance.

The quantification of 5-FU pathway proteins via the AQUA system has numerous advantages over conventional detection methods. For instance, the continuous scores generated by AQUA directly correlate with protein concentrations instead of the

traditional pathologist-based categorical scoring of tissue, which is subjective and subject to human biases. Additionally, AQUA is very high throughput thereby allowing analysis of a large TMA, such as the one in this study, up to 30 to 50 times faster than pathologist based scoring.[12] AQUA also possesses the ability to detect subcellular localization of targets. While the nuclear to cytoplasmic (N/C) protein expression ratios were not significantly associated with clinical resistance phenotype in this study, Gustavon and colleagues found that colorectal cancer patients with higher TS N/C ratios did have a significantly shorter survival.[13] The interpatient heterogeneity was higher than expected based on other published work and likely reflected the inclusion of a third replicate and the molecular diversity among these samples. To counterbalance this, the median expression was chosen instead of the mean, as median would be a better measure of central tendency given the distributions of our data.

All three of the 5-FU pathway proteins that had significant variation in expression between exposed and unexposed tumor samples were consistently linked together in correlation analysis (Table 3.5). This is suggestive of coregulation, and although the mechanistic basis must be elucidated, it is likely associated with these proteins' roles in folate biochemistry. Additionally, the knockdown of the genes encoding these proteins and subsequent increased CRC cellular sensitivity to 5-FU certainly supports these pathway targets involvement in 5-FU resistance.

The primary focus of this study was quantifying the expression for 5-FU pathway proteins in colorectal liver metastases. The molecular heterogeneity of tumors is widely thought to cause CRC resistance to therapy. The protein expression of most targets within

the 5-FU pathway have not previously been systematically quantified in intact tumor tissues.

Technical advances have made quantitative expression profiling of functionally-relevant protein feasible. Ideally, confirmation of these results in a separate validation cohort would have been performed but the requirement that the validation cohort be large to achieve adequate statistical power coupled with the lack of sample availability and other resources limitations made performing validation infeasible. Thus, this study represents an initial investigation into the molecular basis within the 5-FU pathway for clinically-sensitive and resistant patient groups. Future studies will need to correlate the expression of these pathway protein within sensitive and resistant patients with therapeutic outcome. Because 5-FU is one of the most active and frequently used anti-neoplastic agents for CRC, treatment failure caused by its resistance has substantial clinical implications. Knowledge of molecular contributors to resistance may be clinically useful for individualizing patient therapy, as resistant patients could be spared potential toxicity from 5-FU or be given a regimen with a greater potential for efficacy. In summation, this study demonstrates the importance of quantifying the expression of 5-FU pathway proteins in well-phenotyped samples and provides evidence for the underlying molecular differences between 5-FU sensitive and resistant patients.

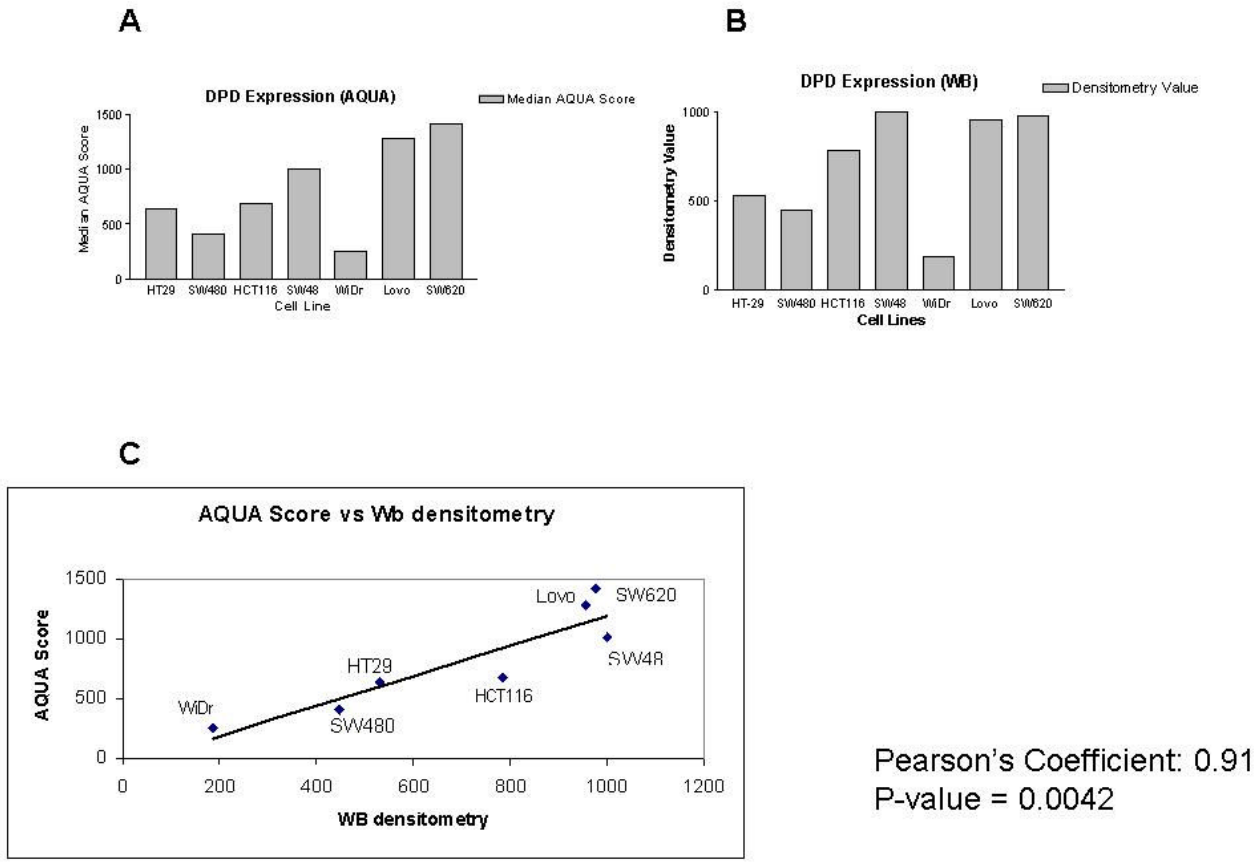


Figure 3.1 Distribution of DPD AQUA scores and western blot densitometry values in cell lines controls. A. Distribution of DPD AQUA scores in 7 cell lines controls embedded into a control cell line array. B. Quantification of DPD bands on Western blot; the dynamic range of DPD expression is consistent with AQUA analysis. C. Correlation of AQUA scores representing and Western blot in the same cell lines for DPD expression.

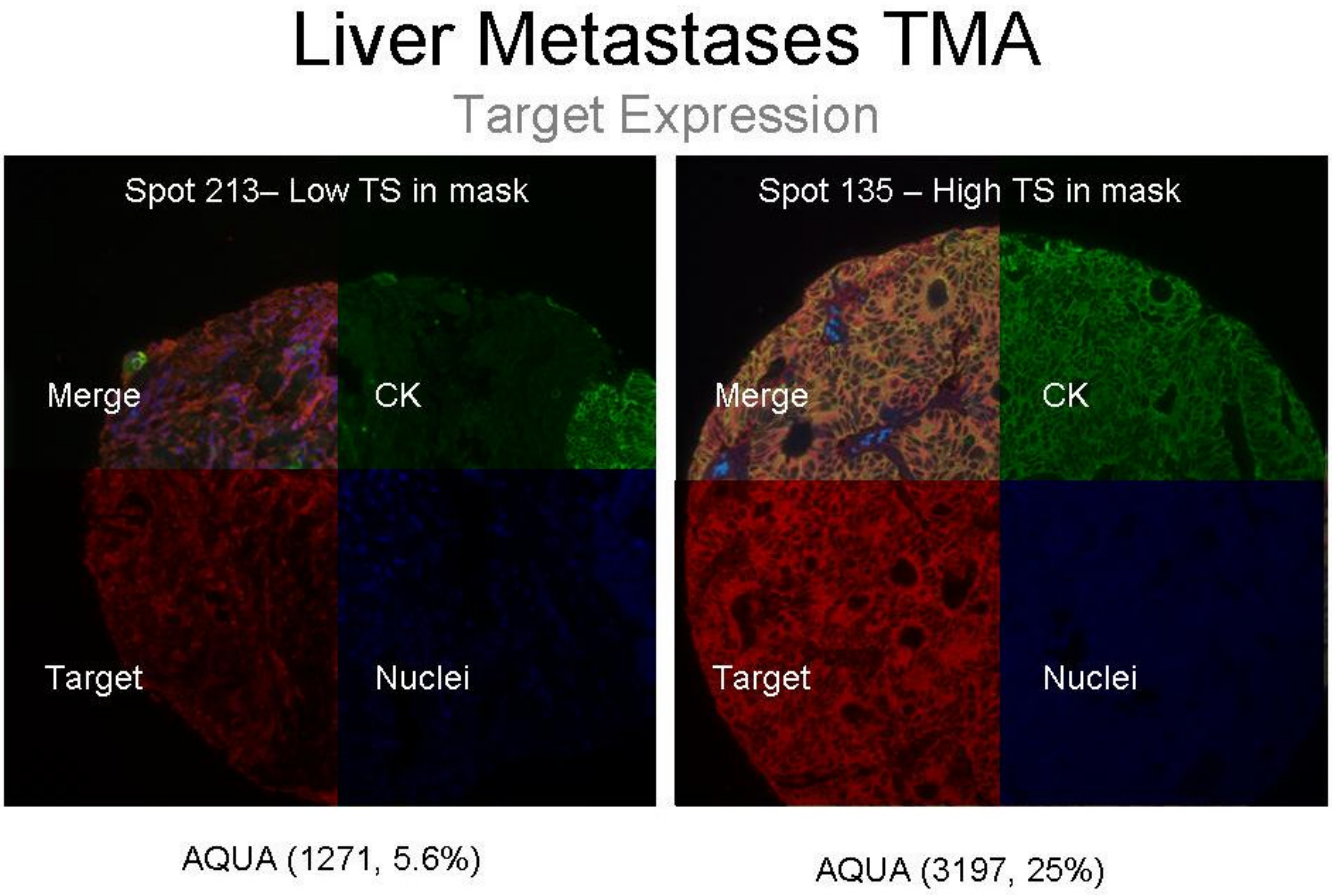
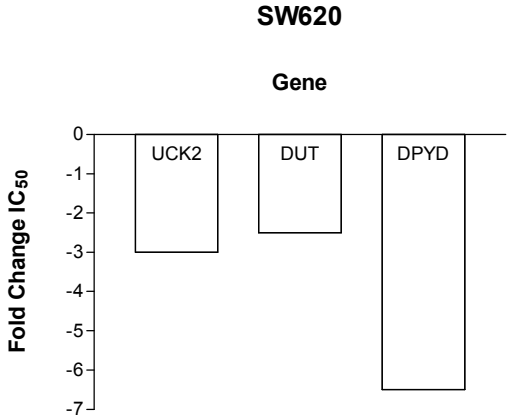
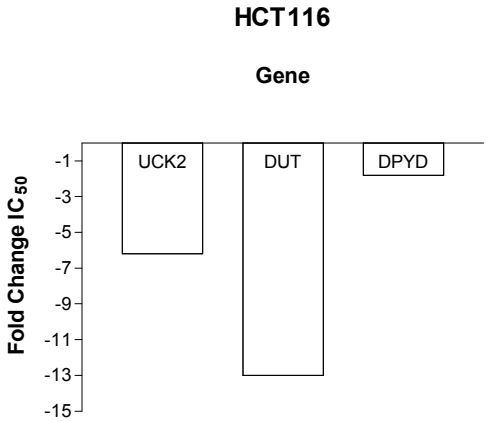


Figure 3.2 Representative AQUA of 5-FU pathway target (TS) in colorectal liver metastases. A. Spot 213 has low target (TS) in tumor mask. B. Spot 135 has high target in tumor mask. CK stands for cytokeratin, nuclei stained with DAPI.





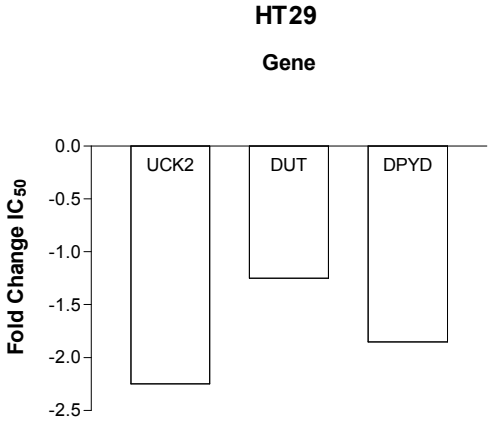


Figure 3.3 Profiles of cell sensitivity of genes encoding proteins whose expression is significantly linked to 5-FU exposure. Waterfall diagrams show fold change in the IC_{50} relative to control eGFP IC_{50} in the 2-3 shRNA per gene producing the same phenotype. Negative values represent fold change in IC_{50} <1. Cell lines utilized were HCT-116 (A), HT-29 (B) and SW 620 (C).

Characteristic	Unexposed	5-FU Exposed	P
Gender			
Male	25 (61%)	18 (38%)	0.054 (NS)
Female	16 (39%)	29 (62%)	
Race/Ethnicity			
White	29 (71%)	38 (77%)	
Black	10 (24%)	10 (21%)	0.77 (NS)
Asian/Other	2 (6%)	1 (2%)	
Age (mean) at diagnosis[1]	64 (range 42 – 84)	58 (range 41– 85)	0.01
Tumor			
1	3	2	
2	6	2	0.18 (NS)
3	18	30	
4	3	1	
Nodes			
0	20	19	
1	13	13	0.65 (NS)
2	6	10	
Metastases			
0	24	24	0.37 (NS)
1	13	21	
Initial No. of Metastases			
1	23	19	
2	10	11	0.35 (NS)
>2	5	9	
Range	1-5	1-14	
Median	1	1.5	

[1]Diagnosis refers to diagnosis of liver metastasis

Table 3.1 Clinical cohort demographics and pathological parameters

Variables	Point Estimate	*P* Value
DPD Expression (AQUA)	1.618 (1.034-2.532)	0.0353
Gender (Female vs. Male)	3.338 (1.283-8.684)	0.0135
Race (Black/Other vs. White)	0.606 (0.213-1.720)	0.3467

Table 3.2 Logistic regression multivariate analysis of clinical features and DPD expression as determined by AQUA to evaluate association with 5-FU exposure with indicated point estimates and P values ($p<0.05$).

Variables	Point Estimate	*P* Value
DUT Expression (AQUA)	1.318 (1.046-1.661)	0.0194
Gender (Female vs. Male)	3.293 (1.282-8.457)	0.0133
Race (Black/Other vs. White)	0.618 (0.217-1.760)	0.3674

Table 3.3 Logistic regression multivariate analysis of clinical features and DUT expression as determined by AQUA to evaluate association with 5-FU exposure with indicated point estimates and P values.

Variables	Point Estimate	*P* Value
UCK2 Expression (AQUA)	1.276 (1.060-1.536)	0.0101
Gender (Female vs. Male)	2.832 (1.111-7.217)	0.0292
Race (Black/Other vs. White)	0.503 (0.173-1.458)	0.2057

Table 3.4 Logistic regression multivariate analysis of clinical features and UCK2 expression as determined by AQUA to evaluate association with 5-FU exposure with indicated point estimates and P values.

Unexposed			
R	DPD	DUTN	UCK2
DPD	1.00	**0.43**	**0.47**
DUTN		1.00	**0.45**
UCK2			1.00

Exposed			
R	DPD	DUTN	UCK2
DPD	1.00	**0.65**	**0.47**
DUTN		1.00	**0.50**
UCK2			1.00

Table 3.5 Correlation matrix of DPD, DUT, and UCK2 for unexposed and the exposed samples. Spearman's rank correlations (r) that were significant are bolded.

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Chapter 4

Copy number determination of 5-Fluorouracil Pathway Targets for Mechanistic Elucidation of Metastatic Colorectal Cancer Resistance

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This chapter was published in part in the European Journal of Cancer and is presented in the style of that journal.

Watson RG, Muhale F, Thorne LB, Yu J, O'Neil BH, Hoskins JM, Meyers MO, Deal AM, Ibrahim JG, Hudson ML, Walko CM, McLeod HL, Auman JT. "Amplification of thymidylate synthetase in metastatic colorectal cancer patients pretreated with 5-fluorouracil-based chemotherapy." Eur J Cancer. 2010. [published online ahead of print August 18, 2010] http://www.ejcancer.info/article/S0959-8049(10)00689-1/abstract].

Abstract

Introduction: Resistance to 5-fluorouracil (5-FU) represents a major contributor to cancer-related mortality in advanced colorectal cancer patients. Genetic variations and expression alterations in genes involved in 5-FU metabolism and effect have been shown to modulate 5-FU sensitivity *in vitro*, however these alterations do not fully explain clinical resistance to 5-FU-based chemotherapy.

Methods: To determine if alterations of DNA copy number in genes involved in 5-FU metabolism impacted clinical resistance to 5-FU-based chemotherapy, we assessed thymidylate synthetase (TYMS) and thymidine phosphorylase (TYMP) copy number in colorectal liver metastases. DNA copy number of TYMS and TYMP was evaluated using real time quantitative PCR (qRT-PCR) in frozen colorectal liver metastases procured from 62 patients who were pretreated with 5-FU-based chemotherapy prior to surgical resection (5-FU exposed) and from 51 patients who received no pretreatment (unexposed).

Results: Gain of TYMS DNA copy number was observed in 18% of the 5-FU exposed metastases, while only 4% of the unexposed metastases exhibited TYMS copy gain (p=0.036). No significant differences were noted in TYMP copy number alterations between 5-FU exposed and unexposed metastases. Median survival time was unchanged in 5-FU exposed patients with metastases containing TYMS amplification and those with no amplification. However, TYMS amplification was associated with shorter median survival in patients receiving post-resection chemotherapy (p-=0.026).

Conclusions: These results suggest that amplification of TYMS is a mechanism for clinical resistance to 5-FU-based chemotherapy and may have important ramifications for the post-resection chemotherapy choices for metastatic colorectal cancer patients.

Introduction

5-Fluorouracil (5-FU) is the backbone of treatment for advanced colorectal cancer, as nearly all patients will receive a 5-FU-containing regimen [1,2]. 5-FU's prominence in advanced colorectal cancer treatment is largely a function of its consistent efficacy throughout its five decades of use [3]. However, one of the biggest challenges for the management of advanced colorectal cancer is 5-FU treatment failure, especially in patients who initially respond but later become resistant. Indeed, the five-year survival of those with metastatic colorectal cancer is less than ten percent [4]. Deaths due to clinically-resistant metastatic colorectal cancer disproportionately account for why colorectal cancer is presently the third leading cause of cancer-related mortality [5].

Numerous investigations into 5-FU resistance in metastatic colorectal cancer have focused on genes within its known pharmacokinetic and pharmacodynamic pathway, such as thymidylate synthetase (TYMS), a key therapeutic target. Advanced colorectal cancers that do not respond to 5-FU-based chemotherapy have greater TYMS enzymatic activity than cancers that do respond [6]. Likewise, liver metastases expressing high levels of TYMS mRNA or protein have also been associated with lack of clinical response to 5-FU *in vivo* [7-10]. A recent meta-analysis of 24 studies has indicated that metastatic colorectal tumors with low expression of TYMS are more sensitive to fluoropyrimidine-based chemotherapy [11].

Similar expression studies have also been performed for thymidine phosphorylase (TYMP) because of its role in 5-FU metabolism. Overexpression of TYMP has been linked to increased 5-FU sensitivity *in vitro* [12]. Xenografts transfected to overexpress

TYMP showed a 43% decrease in size following 5-FU administration, whereas no response to 5-FU was observed in the xenografts lacking TYMP-transfection [13]. In one small study, an increased in TYMP expression was observed in colorectal cancer metastases, which were significantly more resistant to 5-FU than their matched primary tumors [14]. More recently, low TYMP expression was observed to be predictive of response to 5-FU based chemotherapy in metastatic colorectal cancer patients [15]. Conversely, increased expression of TYMP measured by immunohistochemistry was associated with prolonged survival in metastatic colorectal cancer patients treated with capecitabine plus irinotecan [16]. Thus the role TYMP plays in resistance to chemotherapy remains to be clarified.

The underlying mechanism(s) for altered expression of genes and proteins important for drug resistance has implications for the development of strategies to overcome clinical resistance. Variants in the promoter region of genes are one mechanism through which expression can be influenced. A polymorphic tandem repeat sequence in the TYMS gene promoter region is associated with higher TYMS expression [17]. Additionally, colorectal cancer patients with liver only metastases showed a significant association between the high expressing TYMS genotypes and lack of tumor response to 5-FU-based chemotherapy [18]. It also appears that administration of 5-FU-based chemotherapy has the potential to lead to increased expression of TYMS, suggesting a role for acquired resistance to chemotherapy [19]. Alternatively, alterations in DNA copy number are another mechanism of influencing gene expression. Amplification of chromosome 18p11.32, the location of the TYMS gene, was strongly associated with resistance to 5-FU based drugs in human tumor mouse xenografts[20].

TYMS gene copy number has also been associated with clinical resistance to 5-FU. TYMS copy number gains occurred significantly more frequently in liver metastases from patients who had received 5-FU than in metastases from patients who were 5-FU naïve [21]. In addition, patients with TYMS copy number gains also exhibited a 3.5-fold higher relative risk of death than those patients with normal TYMS copy number [21]. However, this small study has not been replicated.

To gain insight into the mechanisms of clinical drug resistance, we examined TYMS and TYMP copy number in resected metastatic colorectal cancer tissue from patients exposed and not exposed to 5-FU-based chemotherapy. Gain of TYMS gene copy was associated with 5-FU exposure suggesting this genetic alteration as one of the mechanisms underlying resistance to 5-FU-based chemotherapy.

Methods

Tissue samples including normal tissues and liver metastases were obtained from colorectal cancer patients undergoing liver resection at the University of North Carolina at Chapel Hill Hospital between 1998 and 2008 by the Lineberger Comprehensive Cancer Center's Tissue Procurement Facility. Clinical information was retrospectively retrieved from patient records and by the Social Security Death Index (SSDI). Tissue specimen analysis and patient chart reviews was approved by an institution review board (IRB number 07-1525) and was performed in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations. Patient samples were categorized as "5-FU exposed" if the patients received 5-FU within the 6-months preceding their liver resections; all other samples were classified as unexposed.

DNA was extracted from frozen liver metastases specimens using the Qiagen All Prep kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol. Briefly, liver metastases were lysed and homogenized via Tissuelyser (Qiagen Inc, Valencia, CA). DNA quality was assessed based on the optical density (OD) 260/280 ratio. DNA extractions were performed at UNC Lineberger Comprehensive Cancer Center's Tissue Procurement Facility.

DNA copy number was determined by quantitative real-time PCR (qRT-PCR) using the ABI 7300. Briefly, qRT-PCR for TYMS consisted of a 20 μL reaction mixture composed of 10 μL of TaqMan universal PCR master mix (Applied Biosystems Inc. – ABI), 10 μL of primer probe mix, and 20 ng of DNA (dried in wells of the PCR plate overnight before adding the reaction mixture), which were performed in triplicate. The

PCR primers and probe for TYMS are: forward primer – GCCTCGGTGTGCCTTTCA, reverse primer – CGTGATGTGCGCAATCATG and probe - CATCGCCAGCTACGCCCTGCTC. The colorectal cancer cell line, H630R10 (kindly provided by Prof Patrick Johnston, Queen's University, Belfast), was used as a control as it exhibits TYMS copy number gain [22]. The qRT-PCR assay for TYMP copy number required a 20 μL reaction mixture composed of 10 μL of SYBR Green (Applied Biosystems Inc. – ABI), 10 μL of PCR primers for TYMS, and 20 ng of DNA (dried in wells of the PCR plate overnight before adding the reaction mixture), which were performed in duplicate. The PCR primers for TYMS are: forward primer – GTTCTCCATTGTCTCCAACCTC and reverse primer – AACTTAACGTCCACCACCAGAG [23]. DNA copy number for each sample was determined by using the delta delta Ct method, in which the threshold cycle (Ct) numbers were calculated by using ABI7300 software. For both the TYMS and TYMP copy number assay, DNA copy number was normalized to RNaseP because normal liver has two copies of RNaseP [23]. The primers for RNaseP are: forward primer - TGGGAAGGTCTGAGACTAGGG and reverse primer - CGTTCTCTGGGAACTCACCT.

The range of values for normal TYMS and TYMP were determined by performing the assays on cell lines from the Centre d'Etude du Polymorphisme Humain (CEPH) collection, which are lymphoblastoid cells taken from healthy individuals believed to have normal TYMS and TYMP copy number. Metastases samples with copy number values more than 2 standard deviations greater than the mean CEPH copy number (2.53 for TYMS, 2.54 for TYMP) were considered to have copy number gains and those with

values more than 2 standard deviations less than the mean CEPH copy number (1.31 for TYMS, 1.52 for TYMP) were considered to have copy number losses. Based on the total sample size we had the power to detect a 0.15 difference between exposed and unexposed groups.

The proportion of the different demographic characteristics in the 5-FU exposed and unexposed patients were compared using either Fisher's exact test or Chi Square likelihood ratio depending on the number of comparison being made. The Fisher's exact test was used to test significant association between copy number alterations and exposure groups (5-FU exposed versus unexposed). Kaplan-Meier analysis was performed to test the effect of genetic alterations on overall survival and significance was assessed using log rank tests. Overall survival data was calculated from the date of diagnosis of the liver metastasis to the data of death or last follow-up. Data were censored when patients were lost to follow-up. All statistical test were 2-sided and the significance level was set at $p<0.05$.

Results

Demographics: The study population comprised of 113 metastatic colorectal cancer patients (62 5-FU exposed and 51 unexposed). Their ages from the time of diagnosis of liver metastases ranged between 38 and 86 (mean age of 62). This cohort was comprised of fifty-eight male patients and fifty-five female patients. The racial distribution of patients in this trial was: White, n = 84; Black, n =25; and Asian/Other, n=4. Other patient characteristics are listed in Table 4.1. The mean age at diagnosis in the unexposed group was 6 years greater than in the 5-FU exposed group (p=0.01); no other statistically significant differences in demographic data were observed between the two groups.

TYMS: TYMS DNA copy number was obtained for 111 samples (62 5-FU exposed and 49 unexposed). The values of TYMS copy number ranged from 1.06 to 3.80 for the 5-FU exposed samples and 1.12 to 3.28 for the unexposed samples. The median copy number was 1.89 for exposed samples and 1.78 for unexposed samples. Approximately twice as many metastases had TYMS copy number alterations for the 5-FU exposed group (23%) compared to the unexposed group (10%), although the proportion was not statistically significant (p=0.13). TYMS loss was infrequent in both 5-FU exposed and unexposed groups (3 samples for each group), however the 5-FU exposed samples did have a greater incidence of TYMS amplification compared to unexposed samples (18% vs. 4%, p=0.036; Figure 4.1).

TYMP: TYMP DNA copy number was obtained for 99 samples (54 5-FU exposed and 45 unexposed). The values of TYMP copy number ranged from 0.93 to 6.78

for the 5-FU exposed samples and 1.06 to 6.98 for the unexposed samples. The median copy number was 1.97 for 5-FU exposed samples and 2.00 for unexposed samples. A greater percentage of metastases samples exhibited TYMP copy number alterations than TYMS copy number alterations, but the prevalence of TYMP copy number alterations was similar in the two groups (37% for 5-FU exposed and 42% for unexposed metastases; p=0.68). Amplification of TYMP was roughly equivalent in both treatment groups (20% for 5-FU exposed and 29% for unexposed; p=0.47), as was loss of TYMP (17% for 5-FU exposed and 13% for unexposed; p=1.0).

Overall there were 7 tumor samples that exhibited copy number alterations for both TYMS and TYMP. From the 5-FU exposed group, two samples had copy number loss for both genes, two samples had copy number gains for both genes and one sample had a gain of TYMS copy coupled with a loss of TYMP copy. From the unexposed group, one sample had copy number loss for both genes and 1 sample had copy number gains for both genes.

Since amplification of TYMS was observed in patients exposed to 5-FU-based chemotherapy prior to surgical resection of their tumors, we compared the overall survival times of patients with TYMS gains to patients with normal TYMS. Kaplan-Meier analysis indicated no statistical difference between patients with normal TYMS copy number and those with TYMS gain (Figure 4.3: Median survival for normal copy = 2.52 years, TYMS gain = 2.11 years, p=0.13, log rank test). However, for patients receiving chemotherapy after surgical resection of their metastases, TYMS gain was associated with a poorer median survival of 2.11 years, as compared to 3.61 years in

patients with normal TYMS copy number (Figure 4.3: p=0.026, log rank test). Copy number alterations for TYMP did not alter overall survival time in patients receiving chemotherapy before or after surgical resection of their tumors (data not shown).

Discussion

Aneuploidy is common phenomena in cancer which can have pharmacologic impact if the altered chromosomal regions contain genes important for modulating drug response. Trisomies are common in acute lymphoblastic leukemia cells [24] and chromosomal gains that encompass the pharmacologically important genes TPMT and GGH have been associated with higher activity of the encoded proteins in leukemic cells, which would be expected to impact the clinical response to mercaptopurine [25]. In breast cancer, amplification of ERBB2 (also known as HER-2/neu) is found in a subset of tumors, which has prognostic importance [26] and is associated with decreased response to tamoxifen [27]. In non-small-cell lung cancer, amplification of EGFR is associated with better response to the EGFR tyrosine kinase inhibitor, gefitinib [28]. Thus one mechanism for drug resistance appears to include copy number alterations of pharmacologically important genes in cancer tissue which can impact drug response [25].

Previously, copy number gain of the gene TYMS was observed in a small number of colorectal metastases following exposure to 5-FU, which was associated with worse outcomes [21]. Our analysis of gene copy number indicates a significant association between exposure to 5-FU-based chemotherapy and amplification of the TYMS gene in surgically resected metastatic colorectal lesions, consistent with the aforementioned results. However, while Wang et al only noted TYMS gain in tumors exposed to 5-FU [21], our results suggest that amplification of TYMS in metastatic colorectal cancer can occur in the absence of 5-FU exposure. This phenomenon might indicate that metastatic colorectal tumors can contain genetic alterations that render them intrinsically less sensitive to 5-FU-based chemotherapy. Alternatively, since data on adjuvant

chemotherapy for primary disease was not available for most of this patient cohort, it is possible that the patients received 5-FU-based chemotherapy regimen for their primary disease before the onset of metastasis. It is unknown whether exposure to 5-FU induces amplification of the chromosomal region containing the TYMS gene or if 5-FU chemotherapy preferentially targets cancer cells without the amplification, which results in the survival of resistant cells with TYMS amplification. However, it has been shown that expression of TYMS increases after bolus exposure to 5-FU *in vivo* [19]. Additional work needs to conducted to determine the mechanism for the observed amplification of TYMS in colorectal liver metastases following exposure to 5-FU.

The importance of TYMS amplification for metastatic colorectal cancer patients was illustrated by previous results that indicated TYMS amplification resulted in shorter survival times in patients pretreated with 5-FU [21]. Our dataset cannot directly address this issue. The patient cohort examined in the Wang et al study, were treated with only 5-FU, as this was the standard of care therapy at that time in history [21]. However, in those patients treated with adjuvant chemotherapy following surgical resection of their metastases, median survival time was 1 ½ years shorter in patients with tumors containing TYMS amplification than those with normal TYMS copy number (Figure 4.3B). Thus it appears that in resected tumors containing TYMS amplification that any remaining cancer cells also contain amplified TYMS and thus are resistant to subsequent regimens of 5-FU-based chemotherapy. Furthermore, TYMS copy number alteration could potentially serve as a biomarker for clinical resistance to 5-FU based adjuvant chemotherapy in metastatic colorectal cancer patients. If validated in clinical trials,

TYMS copy number gain in resected tumors could be used to indicate which patients should receive an adjuvant chemotherapy regimen devoid of 5-FU due to resistance.

The cause of 5-FU resistance in metastatic colorectal cancer patients is believed to be multifactorial [6,29-32]. TYMS amplification clearly does not fully explain resistance to 5-FU based chemotherapy, as some patients without gain of TYMS also had short survival times. A pathway-based approach to interrogate all the genes and proteins that are postulated to be involved in 5-FU metabolism and efficacy is a more likely strategy to identify the relevant mechanisms underlying clinical resistance to 5-FU. While our data does not support copy number alterations in TYMP to be involved in resistance to 5-FU based chemotherapy, there are still many other genes that need to be investigated. Copy number variation assessment within dihydropyrimidine, deoxyuridine triphosphate, and uridine cytidine kinase were not evaluated despite their protein expression being significantly linked to 5-FU exposure because their mRNA expression failed to show significant differences between these exposed and unexposed patients (data not shown). Other genes were not evaluated for this analysis since the other pathway targets evaluated (NME1, RRM1, RRM2) did not have differential expression at the protein level. In addition, the problem of clinical resistance has become more complicated by the additional chemotherapeutic agents that are utilized with 5-FU in metastatic colorectal cancer patients. Detailed investigation of the role played by genes relevant to each of the chemotherapy agents used will provide a more complete picture of the mechanism(s) underlying clinical resistance to complex drug regimens. In addition, since our knowledge of the mechanisms through which these drugs exert their anticancer effects is incomplete, an unbiased approach, such as through genome-wide profiling, may be

required to fully elucidate the mechanisms underlying clinical resistance in metastatic colorectal cancer patients.

In conclusion, we found neoadjuvant treatment with 5-FU-based chemotherapy was associated with gain of TYMS gene copy number. In addition, patients with tumors containing TYMS amplification exhibited significantly shorter overall survival time when treated with chemotherapy following surgical resection of their metastases. These data, along with previously published results, implicates TYMS amplification in clinical resistance to 5-FU-based chemotherapy in metastatic colorectal cancer patients. If validated in larger clinical trials, these results suggest a prognostic importance for TYMS copy number gain in metastatic colorectal cancer patients, which can assist in the selection of the chemotherapy regimens most likely to be of clinical benefit.

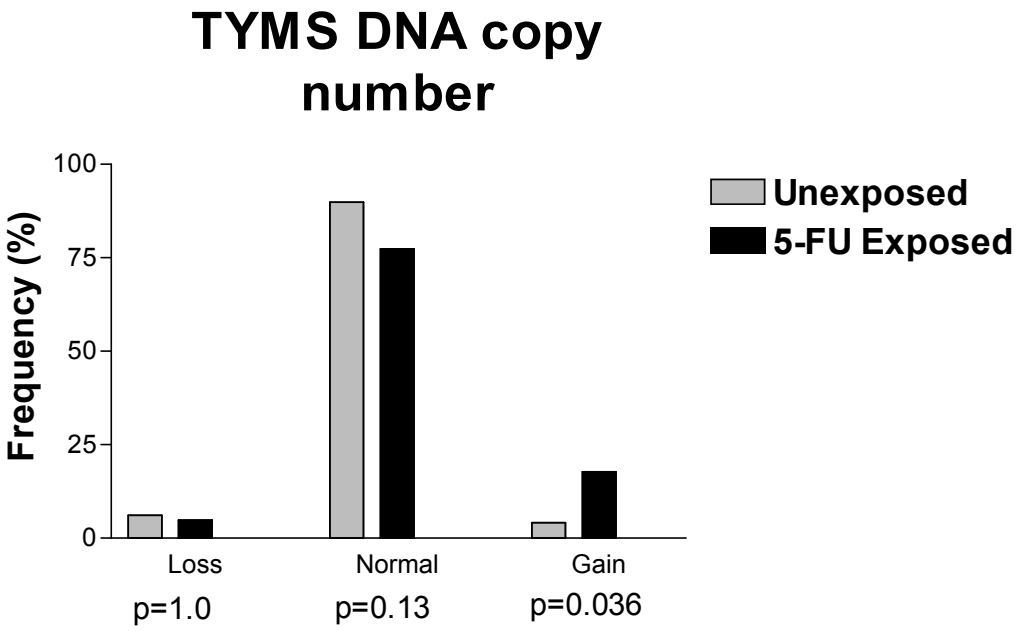

Figure 4.1 Histogram of TYMS copy number in tumors from 5-FU exposed and unexposed patients. Normal DNA copy number ranged from 1.31 – 2.53; tumors with copy number < 1.31 were classified as loss of copy number and tumors with copy number > 2.53 were classified as gain of copy number. The p-value corresponds to the Fisher's Exact Test.

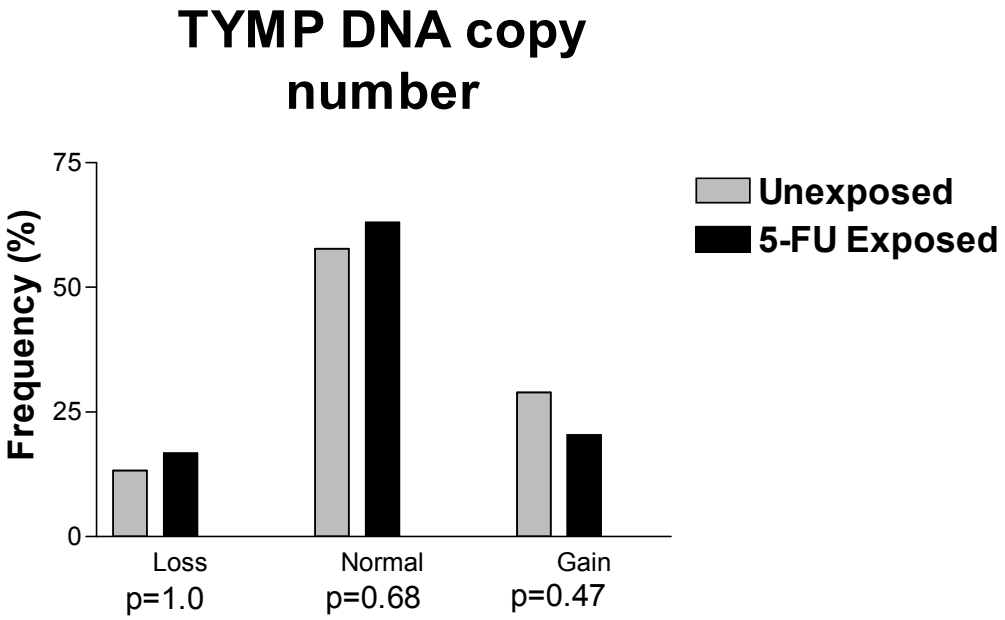

Figure 4.2 Histogram of TYMP copy number in tumors from 5-FU exposed and unexposed patients. Normal DNA copy number ranged from 1.52 – 2.54; tumors with copy number < 1.52 were classified as loss of copy number and tumors with copy number > 2.54 were classified as gain of copy number. The p-value corresponds to the Fisher's Exact Test.

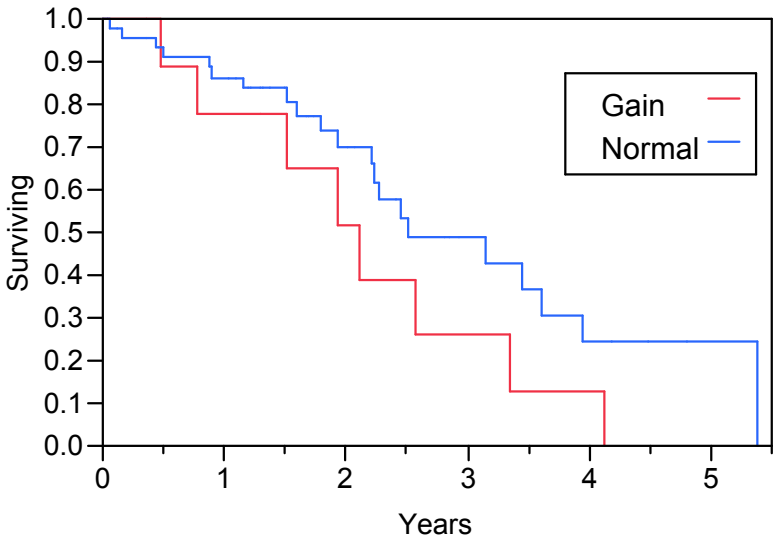

Figure 4.3 Effect of TYMS amplification on overall survival in metastatic colorectal cancer patients receiving chemotherapy. Kaplan-Meier analysis of overall survival in patients with metastatic colorectal cancer receiving neoadjuvant chemotherapy prior to surgical resection of their metastases (median survival time for TYMS amplification = 2.11 years, median survival for normal copy = 2.52 years, p=0.13, log rank test).

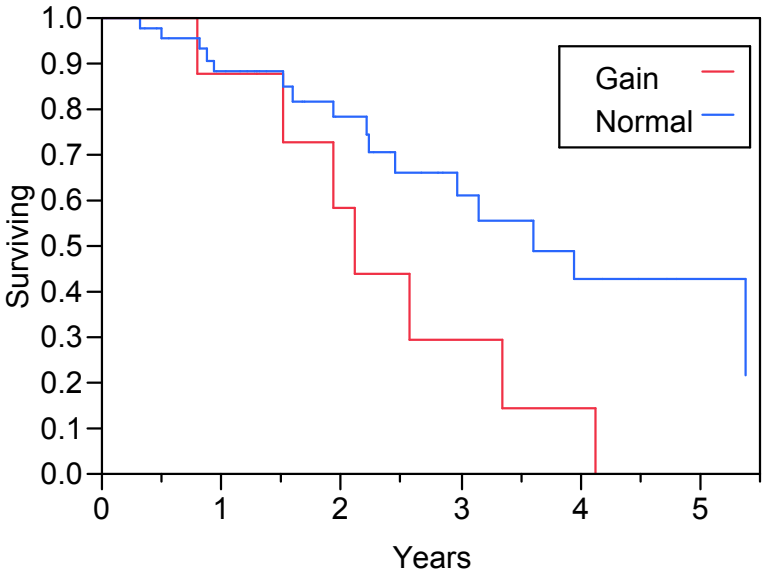

Figure 4.4 Effect of TYMS amplification on overall survival in metastatic colorectal cancer patients receiving chemotherapy. Kaplan-Meier analysis of overall survival in patients with metastatic colorectal cancer receiving adjuvant chemotherapy following surgical resection of their metastases (median survival time for TYMS amplification = 2.11 years, median survival for normal copy = 3.61 years, p=0.026, log rank test).

Characteristic	Unexposed	5-FU Exposed	P
Gender			
Male	31 (61%)	27 (44%)	0.089
Female	20 (39%)	35 (56%)	
Race/Ethnicity			
White	36 (71%)	48 (77%)	
Black	12 (24%)	13 (21%)	0.53
Asian/Other	3 (6%)	1 (2%)	
Hispanic	0	1 (2%)	
Age (mean) at diagnosis[1]	65 (range 42 – 86)	59 (range 38-85)	0.01
Tumor			
0	12	10	
1	5	2	
2	7	5	0.15
3	22	38	
4	3	1	
Nodes			
0	27	25	
1	14	17	0.46
2	8	14	
Metastases			
0	30	28	0.091
1	17	31	
Initial No. of Metastases			
Unknown	8	12	
1	26	23	
2	11	12	0.37
>2	6	14	
Range	1-6	1-14	
Median	1	1	
Length of Neoadjuvant Chemotherapy (days)			
Mean		140	
Median		110	
Range		27-564	

[1]Diagnosis refers to diagnosis of liver metastasis

Table 4.1 Clinical cohort demographics and baseline disease characteristics

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Chapter 5

Molecular Heterogeneity and 5-Fluorouracil Resistance in Metastatic Colorectal Cancer

Patients often respond to medication differently. There are a myriad of reasons causing variable drug effects including: the nature and severity of the disease, an individual's age, organ function, drug interactions, and other diseases.[1] Despite the importance of these factors, genomic, proteomic, and other molecular differences in metabolism, disposition, and the targets of drug therapy can have an even greater impact on overall drug response.[2] There have been numerous investigations into the molecular etiologies of variable drug response, particularly resistance to antimicrobial and antineoplastic agents.[3] As the third most commonly used chemotherapeutic agent, 5-FU resistance has been the subject of many of these studies.[4-7] Still, the underlying mechanisms surrounding 5-FU resistance remain elusive. Ultimately, executing more rigorous investigations, selecting biologically-relevant approaches to target discovery, utilizing tools with greater sophistication for analyses, and choosing the appropriate patient cohorts meticulously are necessary to gain better insight into the causes of 5-FU resistance.

One of the impediments to cancer resistance research is that many studies evaluating 5-FU resistance lack methodological standardization. Typically this occurs because the ideal approach to evaluate a particular molecular target has yet to be determined. Unfortunately, this lack of standardization often limits the predictive value for any biomarker studied. For example, 5-FU pathway target, thymidylate synthase has been proposed as a predictive marker for 5-FU efficacy; however, to date, there is no standardized assay for measuring it. There is little consistency between the thymidylate synthase macromolecules (DNA, mRNA or protein) chosen across investigations.

Significant heterogeneity exists between different macromolecules, which also explains some of the discordant results. Measuring one form of the thymidylate synthase macromolecule does not provide correlative information about the levels of other thymidylate synthase macromolecules. More specifically, while the cell converts DNA to mRNA that is translated into protein, changes at the DNA copy level do not elicit proportional changes in mRNA and changes at the mRNA expression level are not predictive of the resulting protein expression levels. Importantly, thymidylate synthase protein auto-regulates its own expression, further complicating correlations between thymidylate synthase protein, mRNA, and DNA. Thus, thymidylate synthase protein and mRNA levels are not expected to have strong concordance. Clearly, incorporating such knowledge of drug regulation, metabolism and disposition into the design of pharmacogenomic studies is important and can affect the ultimate interpretation of results.

There are other methodological limitations that confound the utility of 5-FU resistance studies. The sensitivity and predictive value of 5-FU response can vary profoundly based on the selected detection method.[8] For example, thymidylate synthase levels measured by the more quantitative PCR-based methods were found to have greater predictive value for 5-FU response compared to levels measured by IHC.[9] Also, traditional pathologist-based scoring of protein expression via immunohistochemical (IHC) staining is semi-quantitative (categorical), and often suffers from lack of reproducibility, low sensitivity, and low dynamic range.[10] Additionally, the type of tumor material measured (metastasis versus primary) may also influence the prognostic utility of a biomarker. For instance, there are conflicting results published on thymidylate

synthase expression in metastases and primary tumors.[11-13] A recent meta-analysis found that if thymidylate synthase expression was determined in the metastatic lesion, the hazard ratio (HR) was 2.39 (95% CI, 1.43–4.01) compared to only 1.33 (95% CI, 1.07–1.61) when measured in the primary cancer.[9]

As mentioned earlier, selecting biologically-relevant approaches to choosing targets is critical for exploring mechanisms of 5-FU resistance. While many studies focus on the single gene candidate approach to identifying resistance mechanisms of 5FU, a pathway-guided target approach may actually be more beneficial for several reasons. This approach simultaneously investigates the interaction of targets critical to 5-FU activation, catabolism, and therapeutic activity. Although single candidate studies do contribute to the aggregate knowledge of 5-FU pharmacokinetic and pharmacodynamics, their focused scope limits their ability to capture the complex interplay between 5-FU pathway targets and its resistance. With 24 proteins comprising the 5-FU pharmacokinetic and pharmacodynamic pathway known to date, it is quite improbable that 5-FU resistance would be wholly attributable to one target. Thus, an approach that investigates multifactorial etiologies of 5-FU resistance could provide a more complete characterization.[7] Additionally, a pathway-guided approach employs rigorous interrogation of targets that have pharmacodynamic and pharmacokinetic data supporting their relevance to 5-FU. Also, investigating a small number of pathway targets in a limited number of samples often has more statistical power to detect targets with small effect sizes than a genome-wide association (GWA) study because GWA studies typically have substantial correction for multiple hypothesis testing.[14] Moreover, using *a*

priori knowledge to investigate target heterogeneity *in situ* or within clinical samples allows for clinical relevance to more directly be established.

The phenotype of the samples investigated can also contribute to the molecular diversity. For example, both normal human polymorphisms and tumor genetic heterogeneity contribute to the large interpatient variability in response to cytotoxic agents. The combined effects of genetic heterogeneity of tumors and normal human polymorphisms are in part responsible for the large interpatient variability existing for nearly all targeted and cytotoxic agents. These molecular changes significantly impact 5-FU metabolism and disposition *in vivo*. Phenotyping tumors based on clinical resistance (exposure) to 5-FU increases the ability to identify the underlying contributors to resistance through the process of enrichment. Enrichment refers to the belief that exposed samples are uniquely concentrated with molecular patterns that allow tumorigenesis to persist despite adequate 5-FU therapy. Evidence for this phenomenon has been published.[15,16]

The central aim of this dissertation project is to use a pathway-guided approach to identify mechanistic determinants of 5-FU resistance in metastatic colorectal cancer patients. This objective was addressed two parts. The first section of this dissertation characterized 5-fluorouracil pathway targets whose expression differed in clinical resistant (exposed) colorectal liver metastases compared to unexposed tumors. Specifically, patients were phenotyped for 5-FU exposure and various clinicopathological variables, samples were incorporated into a tissue microarray, and the protein expression was quantified using AQUA. This step required the construction of a TMA and the

retrospective analysis of patient charts and pathological reports. The second part of this project investigated the mechanism whereby 5-FU pathway targets associated with 5-FU exposure cause resistance (again using colorectal liver metastases). Copy number variants in genes encoding targets from the 5-FU pathway were identified that were associated with 5-FU exposure. Additionally, shRNA-mediated knockdown of genes encoding UCK2, DPD, and DUT, the pathway proteins with expression significantly associated with 5-FU exposure, was undertaken to see if loss of these genes would augment cellular sensitivity to 5-FU. This combined-modality approach allows for the characterization of the molecular determinants for 5-FU resistance.

The goal of this concluding chapter is to assimilate the findings of this dissertation project, to discuss these findings within the context of the broader body of scientific literature, and to evaluate the impact of these findings and potential areas for future investigation.

Summary

Chapter 2

The objective of Chapter 2 to characterized the entire clinical cohort of metastatic colorectal cancer patients stratified by 5-FU exposure phenotype. To this end, 67 exposed patients (received 5-FU within the 6 months preceding their liver resection) and 54 unexposed patients were evaluated with respect to clinical demographics, baseline disease characteristics, chemotherapy, and survival. There were no significant differences with respect to baseline characteristics with the exception of age at diagnosis of liver metastases; the unexposed patients were 6 years older ($p=0.01$). The median overall

survival from surgical resection of colorectal liver metastases was 885 days and the median disease free survival was 294 days.

In addition to 5-FU, thirty-one (46%) exposed patients received oxaliplatin as part of their regimen. Twenty-four (36%) patients received bevacizumab. Fourteen (21%) patients received irinotecan. One (1.4%) patient received cetuximab. Patient chemotherapy regimens, demographics, and disease characteristics reflected the real-world diversity and complexity seen in metastatic colorectal cancer patients and made them well-suited to investigated 5-FU resistance.

Chapter 3

In Chapter 3, the goal was to identify 5-FU pathway proteins with altered expression that is significantly associated with clinical resistance to 5-FU. Patients were grouped based on their 5-FU exposure (in same manner as Chapter 2). Deterioration, "checked out samples," and loss samples dropped the exposed sample size from 67 to 47 and the unexposed sample size from 54 to 41. Cores from the available samples were arranged on a tissue microarray and AQUA analysis of eight important 5-FU pathway targets was performed to quantify protein expression. AQUA algorithms allow for continuous, rapid, unbiased, sensitive detection of targets within subcellular compartments of cells. In the univariate analysis, UCK2 was the sole target that had a significantly different expression pattern between the exposed and unexposed samples. When using a multivariate analysis model that adjusted for race and gender, UCK2 (0.019), DPD ($p=0.035$), and DUT ($p=0.0135$) had differential expression between exposed and the unexposed. The model was very sensitive to gender secondary to target expression. Additionally, shRNA knockdown of DPD, DUT, and UCK2 in 3 colorectal

cancer cell lines was performed, so that the cellular cytotoxicity phenotype in the presence and absence of 5-FU could be determined. Knockdown of these gene did modulate the cellular 5-FU IC_{50}. Knockdown mediated by shRNA resulted in at least a 2-fold increased sensitivity to 5-FU in three cell lines for UCK2 (HCT116, HT29, and SW620), in two cell lines for DUT (HCT116 and SW620), and in one cell line for DPD (SW620). DPD was just below the threshold for credentialing (2-fold change in 5-FU IC_{50}) for both HT29 and HCT116.

To corroborate the validity of AQUA data, its variability, and its predictive power, validation in an independent population is typically performed. We investigated using a paired sample approach to validate our findings and concluded that including a validation cohort for this study was impractical due to lack of our ability to achieve adequate statistical power and limited resources. Accordingly, the feasibility of using patients' pre and post 5-FU exposure samples as a validation cohort was determined. Approximately 25% of the exposed patients also had unexposed tumors, biopsy samples taken prior to exposure, banked at UNC. This meant that there are approximately 12-16 matched samples from the retrospective cohort that could be used for AQUA. Before using the 12-16 matched samples as a validation cohort, I determined how much target expression must differ between the exposed and pre-exposed samples to have adequate power.

Assuming 12 matched samples are available (n=12), low inter-patient variability (0.06 SD), and comparing each patient's ratio of expression in his 5-FU exposed sample to the expression in his unexposed (biopsy) sample, there is 35% power to detect a mean

difference of at least 1.3. Thus, if the effect size is similar to what was seen in the initial AQUA analysis (Chapter 3), there is little power to detect a difference between exposed versus unexposed samples. If the effect size of 5-FU exposure was noticeably larger, then the power is more reasonable, even with high variability between patients. For example, assuming n=12, high inter-patient variability (1 standard deviation), and comparing each patient's ratio of expression in her 5-FU exposed sample to the expression in her unexposed (biopsy) sample, then there is 71% power to detect a mean difference of at least 1.8.

These analyses suggest that constructing a validation cohort appears technically feasible, assuming the same incidence of biopsies banked at UNC (25%) and assuming that these samples are indeed in the tissue procurement facility bank. Deterioration, "checked out samples," and loss samples previously dropped the exposed sample size from 67 to 47 (Chapter 3). While technically feasible, it also appears statistically unlikely that this validation cohort will be able to replicate the previous results because of two reasons: small sample size (n=12-16) and a modest effect size (based on the initial analysis in protein, Chapter 3). Of course, with patients serving as their own controls, the 5-FU effect size could improve. Still, even with a generous effect size of 1.8, the study remains underpowered.

Chapter 4

In Chapter 4, the primary aim was to determine whether gene copy number was associated with 5-FU exposure of pathway targets. Accordingly, patients were grouped based on their 5-FU exposure. DNA was isolated from patient colorectal liver metastases that were acquired during surgical resection. Gene copy number assessment was

performed for thymidylate synthase (TYMS) and thymidine phosphorylase (TYMP) in the overall clinical cohort (Chapter 2). Gain of TYMS DNA copy number was observed in 18% of the 5-FU exposed metastases compared to only 4% in the unexposed metastases group (p=0.036). TYMP copy number was not associated with significant differences between 5-FU exposed and unexposed metastases.

These findings indicate that amplification of TYMS is a mechanism for clinical resistance to 5-FU-based chemotherapy and may have important implications for the post-resection chemotherapy choices for metastatic colorectal cancer patients. Copy number variation assessment within DPD, DUT, and UCK2 were not evaluated despite their protein expression being significantly linked to 5-FU exposure because their mRNA expression failed to show significant differences between these exposed and unexposed patients (data not shown). Other genes were not evaluated for this analysis since the other pathway targets evaluated (NME1, RRM1, RRM2) did not have differential expression at the protein level.

An underlying hypothesis explored in this dissertation project is that copy number variants elicit altered downstream expression. Thus, evaluating mRNA expression and gene copy number for 5-FU pathway targets whose protein expression were associated with 5-FU resistance would provide an unambiguous mechanism by which the molecular heterogeneity occurs. AQUA was used as a filter for identifying pathway targets with a functionally relevant association with 5-FU resistance. The mRNA expression data were evaluated for DPD, DUT, and UCK2, which are the three 5-FU pathway proteins identified as significant via AQUA. Gastrointestinal Spore collaborator, Jen Jen Yeh

M.D., provided gene expression data using a microarray for 24 liver metastases exposed to 5-FU and 20 unexposed liver metastases. Although DPYD, UCK2, and DUT were part of her screen, their expression was not differentially expressed based on 5-FU exposure. Since Dr. Yeh utilized the same samples used in the retrospective cohort (Chapter 2) developed for this dissertation and had the same study design, her results were highly prejudicial for not performing additional mRNA studies for these samples and targets. Additional mRNA expression studies would be unlikely to yield different results in spite of a somewhat larger sample size.

There are numerous potential reasons as for why the mRNA and protein expression data didn't correlate including: negative feedback (proteins directly and indirectly regulate their own gene expression through multiple mechanisms), post-translational regulation (such as methylation), mutations in exon or promoter regions, and mutated gene products with altered degradation rates. Due to the sheer abundance of potential explanations for the discordance, it was impractical to continue exploring correlations and beyond the scope of this project. Since mRNA expression data failed to demonstrate a significant difference between exposed and unexposed samples, copy number assessment for UCK2, DPYD, and DUT was not performed. Note copy number evaluation of TYMS and TYMP were conducted as part of an exploratory analysis before the decision was made to use AQUA to screen targets for functional relevance. Additionally, there were poor correlations between thymidylate synthase (gene and protein, Spearman's -0.0170, p=0.8777) and thymidine phosphorylase (gene and protein, Spearman's -0.1829, p=0.1214) for AQUA and real time PCR detection. This lack of gene-protein correlations for thymidylate synthase and thymidine phosphorylase

indirectly provides support for not evaluating copy for other targets. The lack of correlation could result from a myriad of reasons including dysregulation of gene copy and RNA resulting from insertions and deletions and point mutations that alter RNA turnover or cellular senescence. With TS autoregulation of its own expression, discordance is not uncommon.[7] This could also reflect the tumor-specific expression determined by AQUA compared to a mixture of normal and tumor cells present in extracted DNA or mRNA from the metastases.

Future Directions

This dissertation research has prompted a number of proposed future studies into the impact of 5-FU pharmacokinetic and pharmacodynamic pathway proteins on 5-FU resistance. A discussion of the proposed follow-up studies follows.

Target Validation with AQUA

In order to determine the degree to which the AQUA results are applicable to other mCRC patients, the next step would be to validate these results using another TMA composed of colorectal liver metastases. Ideally, a well-matched independent validation cohort would already exist and be available through collaboration, but if none are available then one could be created in a manner similar to the development of the retrospective clinical cohort (see Chapter 2). Note, an independent cohort would be most desirable, as it would help avoid coverage error, which occurs when there is a discrepancy between the target population and the population from which the sample was derived.[17] Note that coverage error potentially compromises the ability to generalize the results of the study. Samples from the validation cohort would be phenotyped based on 5-

FU exposure (as described in Chapter 2). The same inclusion and exclusion criteria and methodologies would be employed as the initial cohort (see Chapters 2 and 3). Also, it would be important for the validation cohort to have similar baseline disease characteristics (in addition to AJCC stage), chemotherapy regimens, and patient demographics. To assess the similarities between the cohorts, which would allow for direct comparisons of data, statistical tests evaluating homogenicity of baseline disease characteristics, demographics, and chemotherapy regimens can be performed.[18] Provided that the samples are sufficiently homogenous, any differences apparent in the cohorts are more likely due to molecular heterogeneity or chance (more rare) instead of confounders. However, if the tests of homogenicity are not significant, then the heterogeneous population of colorectal tumor samples may be too distinct to use for validation.

Just as in the initial cohort, differences in the expression of 5-FU pathway targets within colorectal liver metastases will be correlated with 5-FU exposure. Also, now that there is data regarding the effect size of 5-FU exposure on the expression of these 5-FU pathway targets (see Chapter 3), the number of cases included in the replication cohort would ideally be increased. For example, post-hoc statistical power calculation revealed that the initial analysis was underpowered given the modest effect size (power <0.3). Adequate power will decrease the risks of a false-negative result, make sure that the sample represents the population, and make sure that the effects of clinical resistance on target expression are accurately characterized. The recommended sample size would be at least 620 samples to achieve adequate power based on the UCK2 data (see Chapter 3).[19,20] Note, if the validation analysis is also performed at the translational pathology laboratory at UNC, then one consideration is that there is *a priori* knowledge of the

results from the initial analysis; since AQUA is automated, perhaps perceived researcher bias would be minimized.

There are numerous reasons why a validation cohort is important. Validation cohort help avoid spurious conclusions. For example, Gustavson and colleagues quantified TS within primary colorectal carcinomas using AQUA in two large cohorts. The training and validation cohorts consisted of 599 and 447 tumor samples, respectively.[21] In the training cohort, there was a significant association found between a decreased 5-year disease-free survival (DFS) and increased TS nuclear expression (16% decreased survival [72% to 56%], $p<0.001$), cytoplasmic expression (12% decreased survival [70% to 58%], $p=0.02$), and higher nuclear to cytoplasmic expression ratio (15% decreased survival [66% to 51%], $p<0.001$). However, in the validation cohort only the expression ratio showed a significant association with outcome (time to recurrence), $p=0.03$. Ideally these cohorts would have used the same outcome measure; still, both DFS and time to recurrence have certainly been used successfully as a surrogate for survival in other trials; the lack of correlation likely means that nuclear and cytoplasmic expression's impact on survival was overestimated in the training cohort. Thus, by performing analyses in the validation cohort, there is refinement of the model, and the most important variables can be highlighted. Indeed, subsequent unpublished data from the same group also demonstrated that TS nuclear to cytoplasmic ratios was a better prognosticator for clinical outcome than either nuclear or cytoplasmic expression alone. Another advantage of validation cohorts is that they provide greater confidence in the data, as independent validation of results establishes the scientific rigor. For instance, a recent study by Anagnostou and colleagues demonstrates how when the validation cohort

is chosen correctly (or created carefully) and identical experimental methods employed, AQUA data is highly reproducible.[22] The authors used AQUA to quantify the expression of mammalian target of rapamycin in the cytoplasm of lung adencarcinoma cytoplasm. The expression was remarkably similar (56% and 50% of cases) in the training and independent validation cohorts, respectively. Performing a validation cohort also may provide additional scientific insights. For example, in the Gustavson study, TS nuclear to cytoplasmic expression ratio was added to their Cox multivariate proportional hazards multivariate model multivariate model of survival after it came out as significantly associated with outcome in their both cohorts.[21] Moreover, its inclusion in the model improved the prognostic value of the model over clinical and pathological features alone.

Functional Validation

Additional functional validation would also strengthen my results by providing additional mechanistic insights. Cell proliferation refers to the number of cells that are dividing in a culture. Colorectal cancer, as well as other cancer types, is characterized by hyperproliferation. This dysregulation undoubtedly contributes to its pathogenesis. While cytotoxicity was used to evaluate the functional relevance of the targets, the cytostatic effects of the shRNA to DPYD, DUT, and UCK2 (and of 5-FU) were not analyzed. Additionally, cell cycle perturbation has been linked to 5-FU resistance in resistant colorectal cancer cell lines.[23] Understanding the impact of shRNA and 5-FU resistance on the cell cycle would provide a relevant contribution to our understanding of the underlying molecular mechanisms of 5-FU resistance. Another complimentary study would be to modulate the phenotype of CRC cell lines from chemosensitive to 5-FU

resistant. Thus, reasonable next steps in the functional validation of these targets would be to assess the impact on the distribution of the cell cycle and proliferation in CRC cell lines after shRNA knockdown both in the presence and absence of 5-FU. Also, the functional impact of overexpression of prioritized pathway targets will be evaluated *in vitro*. To this end, I propose the following experiments: flow cell cytometry, enzyme-linked immunosorbent assay (ELISA), and transfecting cDNA vectors overexpressing 5-FU pathway target genes. These experiments would be performed using the three cell lines that were used in the initial functional validation (SW620, HT29, and HCT-116; see Chapter 3).

To characterize the functional impact of shRNA and 5-FU resistance on cell cycle, the aforementioned CRC cell lines should undergo flow cell cytometry. Synchronization of the cell cycles would be necessary by incubating them for 48 hours in medium without fetal calf serum at 37° Celsius at 5% carbon dioxide. Cells would then be incubated: 1) with no shRNA and no 5-FU, 2) with shRNA but no 5-FU, and 3) with shRNA and with increasing concentrations of 5-FU. These "treatments" would be added to the medium. After harvesting, rinsing, and fixating the cells, they would be incubated with detergent Triton-X and stained, such as with propidium iodide in PBS. Then, the stained cells will be analyzed via flow cytometry (Beckton Dickinson, Heidelberg, Germany). Cells from each sample would be counted until 10,000 cells were determined to be in a predefined G1-gate. Then, the cell cycle distribution or the percentage of cells in the G0/G1, S, and G2/M phase, can be evaluated using WinMDI version 2.9 software.

Additionally, the cell proliferation could be evaluated with the cell proliferation ELISA bromodeoxyuridine (BrdU) chemiluminescence assay (Roche Diagnostics, GmbH Manheim Germany). This assay allows for assessment of cell proliferation by quantifying BrdU incorporated into the newly synthesized DNA of replicating cells. The sensitivity and activity of this assay should be comparable to the more traditional [^{3}H]-thymidine-based cell proliferation studies.[24] This assay has been used successfully in colorectal cancer cell lines as well.[25] Again, cells could be incubated: 1) with no shRNA and no 5-FU, 2) with shRNA but no 5-FU, 3) with shRNA and in increasing concentrations of 5-FU. Cells would be cultured in 96-well microtiterplate and pulse-labeled with BrdU. Only proliferating cells incorporate BrdU into their DNA. Cells would be fixated, genomic DNA denatured, allowing the incorporated BrdU to be detected. A secondary perioxidase-conjugated anti-BrdU antibody will locate and bind the BrdU label in DNA. Then a peroxidase substrate, such as Luminol/4-iodophenol, will be used to quantify the bound-anti-BrdU complex. Alternatively, an ELISA could also be used to quantify the expression of molecules that regulate cell proliferation, such as cell division protein kinase 1, cyclin A, cyclin B1, $p21^{Waf1}$, and $p27^{Kip1}$. Again, the cells could be stratified as mentioned earlier to interrogate the impact shRNA and 5-FU resistance are making on cell proliferation.

A third plan to investigate the functional relevance of genes that came out of the AQUA screen would be to use cDNA vectors to overexpress the target genes of interest. For example, cells transfected with overexpressing cDNA vectors and those that have an empty vector could both be treated with 5-FU and their respective cytotoxicities compared. This would allow for an *in vitro* investigation of resistance from an opposite

perspective. For example, since UCK2 is overexpressed in clinically resistant (exposed) sample, transfection of cDNA overexpressing UCK2 should result in a higher 5-FU IC_{50} compared to control. Expression from cells from both pre and post plasmid transfection could also be analyzed via AQUA for confirmation of increased UCK2 levels post transfection. This type of modulation is not possible *in vivo* but provides tremendous mechanistic validation.

Clinical Trial

To provide additional insights to the clinical relevance of these targets, they should be investigated as part of another clinical trial. The study population would still consists of patients with colorectal liver metastases, but those with extrahepatic disease or positive surgical margins would be excluded from this analysis to minimize confounders to the survival analysis.[26-28] The size of this cohort would also be larger, such as at least 620 patients, given the modest effect sizes of UCK2, DUT, and DPD (see Target Validation with AQUA in Future Directions and Chapter 3). Patients would be randomized to exposed and unexposed groups prior to resection of their liver metastases. Again, exposed patients would have received 5-FU within the six months preceding their hepatectomies. However, this time all patients would receive adjuvant 5-FU based therapy post resection in accordance to NCCN guidelines; this therapy would be documented. Additionally, pathological, other baseline disease characteristics, and other clinical data including duration of recurrence (recurrence-free survival), pre and post-surgical carcinoembryonic antigen (CEA) levels, and presurgical chemotherapy would be recorded for each patient.

The resected colorectal liver metastases would undergo microdisection in an effort to minimize the normal cell contamination problem, which is evident in bulk tumor samples. Microdissection allows procurement of specific cells or cellular populations from a histology slide via direct microscopic visualization. There are many types of microdissection ranging from simple mechanical systems to more sophisticated instruments, such as laser capture microdissection and selective ultraviolet radiation fractionation.[29-35] The primary result of performing microdissection would be a more homogenous population of tumor cells to carry forward for the analyses. A tissue microarray would again be constructed with triplicate samples for each patient whenever feasible (see Chapter 3). All samples would be randomly distributed across the array. DNA and mRNA from the samples would also be obtained using the Qiagen All-Prep kit (Qiagen Inc, Valencia, CA). Quantification of the expression of 5-FU pathway targets (especially UCK2, DPD, DPYD, and DUT) in the colorectal liver metastases would be performed with AQUA and real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).

Thus, the study design would consist of enriching samples for variation underlying resistance through stratifying patients based on their 5-FU exposure, identifying molecular variation associated with resistance, and then comparing the disease-free survivals between patients with variation associated with resistance and those who don't. This study structure would represent a more comprehensive approach to evaluating the clinical impact of resistance and would potentially have more perceived external validity since recurrence-free survival is a more widely used endpoint compared to exposure. Additionally, univariate and multivariate models for resistance would be

created. The univariate analysis would determine whether molecular variation as determined by AQUA and Real time qRT-PCR is associated with clinical resistance to 5-FU (exposure) using a two-sided Wilcoxon. Then logistic regression would be used for the multivariate model. Importantly, the larger sample cohort would allow for more variables to be included into the model, such as CEA and recurrence-free survival. Gender would also be included in the model.

The interaction between gender, 5-FU exposure-phenotype, and DPD, DUT, and UCK2 protein expression found in dissertation (see Chapter 3) has prompted some interest in defining the role of gender on biomarker predictive value in metastatic colorectal cancer patients. Gender-related expression differences are not unique to this cohort. For example, Gustavson and colleagues recently found a statistically significant longer disease-free survival associated with female gender in colorectal cancer patients.[21] Longer survival and better response to 5-FU in females is believed to be linked to females having lower DPD expression compared to males thereby making females less resistant due to lower 5-FU clearance.[36,37] However, subsequent studies have not consistently replicated this association with outcome.[38-40] Additional studies evaluating the relationship between gender and 5-FU clearance and DPD activity also have conflicting results.[41-46] Presumably, some of the disparity in data results from methodological differences and tissue preparation between studies. However, even within trials reporting significant gender differences, substantial overlap in values existed between males and females. A literature search did not reveal that gender has previously been implicated in differential DUT or UCK2 expression. If gender turns out to be a

significant prognosticator of recurrence-free survival, associated with exposure-phenotype, then the molecular basis would also have to be explored.

Concluding Remarks

AQUA quantification of pathway proteins in tumors coupled with enrichment has the potential to be a powerful tool for identifying functionally-relevant variation associated with resistance. Protein expression profiles can be compared between clinically resistant and sensitive samples, which can then coupled with mRNA expression, copy number determination and *in vitro* experiments for mechanistic elucidation. These results suggest that samples exposed to 5-FU are both phenotypically and molecularly distinct from unexposed tumors. Furthermore, variation in these pathway targets modulates *in vitro* sensitivity to 5-FU in colorectal cancer cells. With further exploration and refinement, this multi-platform model may be able to identify underlying causes of resistance, associated with protein levels, to novel drugs (based on *in situ* proteomic profiling). This would enable better individualization of care: resistant patients to avoid unnecessary exposure to ineffective therapy and to be treated with drug regimens to which they will more likely respond.

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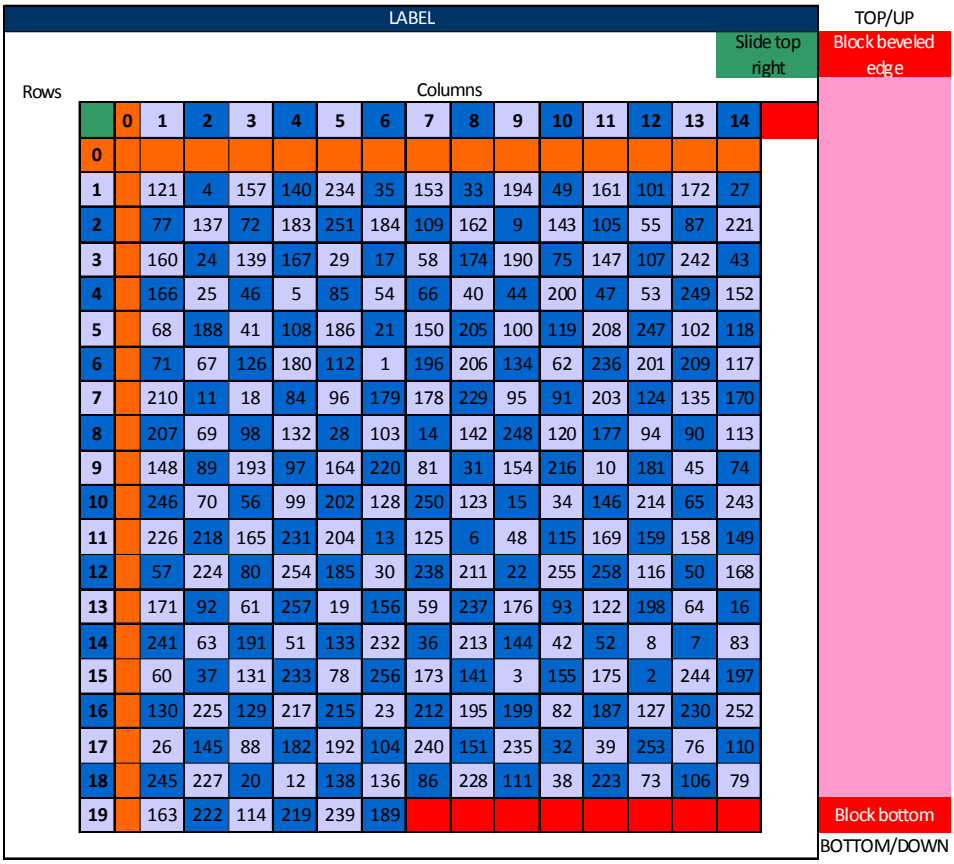
Appendix 1:

Supplementary Data



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2		77	137	72	183	251	184	109	162	9	143	105	55	87	221
3		160	24	139	167	29	17	58	174	190	75	147	107	242	43
4		166	25	46	5	85	54	66	40	44	200	47	53	249	152
5		68	188	41	108	186	21	150	205	100	119	208	247	102	118
6		71	67	126	180	112	1	196	206	134	62	236	201	209	117
7		210	11	18	84	96	179	178	229	95	91	203	124	135	170
8		207	69	98	132	28	103	14	142	248	120	177	94	90	113
9		148	89	193	97	164	220	81	31	154	216	10	181	45	74
10		246	70	56	99	202	128	250	123	15	34	146	214	65	243
11		226	218	165	231	204	13	125	6	48	115	169	159	158	149
12		57	224	80	254	185	30	238	211	22	255	258	116	50	168
13		171	92	61	257	19	156	59	237	176	93	122	198	64	16
14		241	63	191	51	133	232	36	213	144	42	52	8	7	83
15		60	37	131	233	78	256	173	141	3	155	175	2	244	197
16		130	225	129	217	215	23	212	195	199	82	187	127	230	252
17		26	145	88	182	192	104	240	151	235	32	39	253	76	110
18		245	227	20	12	138	136	86	228	111	38	223	73	106	79
19		163	222	114	219	239	189								

Figure A1.1 Configuration of tissue microarray

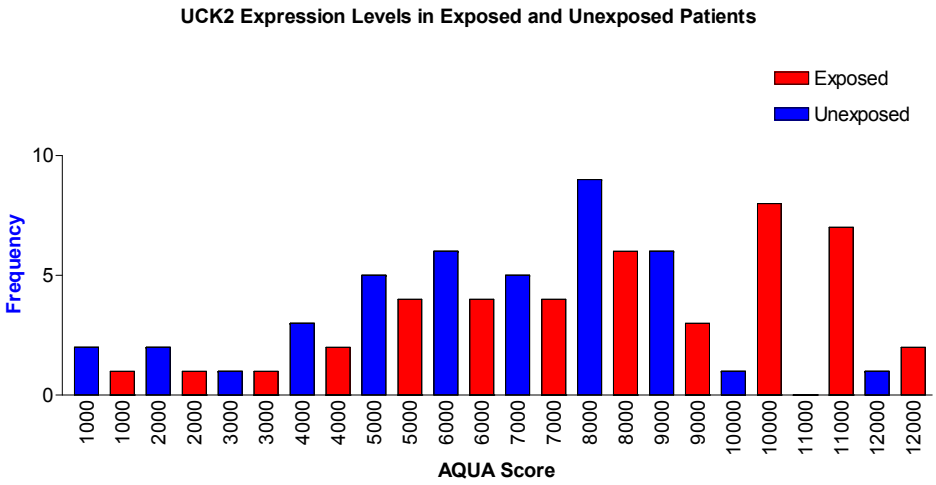


Figure A1.2 Distribution of UCK2 protein expression among the exposed (red) and unexposed (blue) as quantified by AQUA

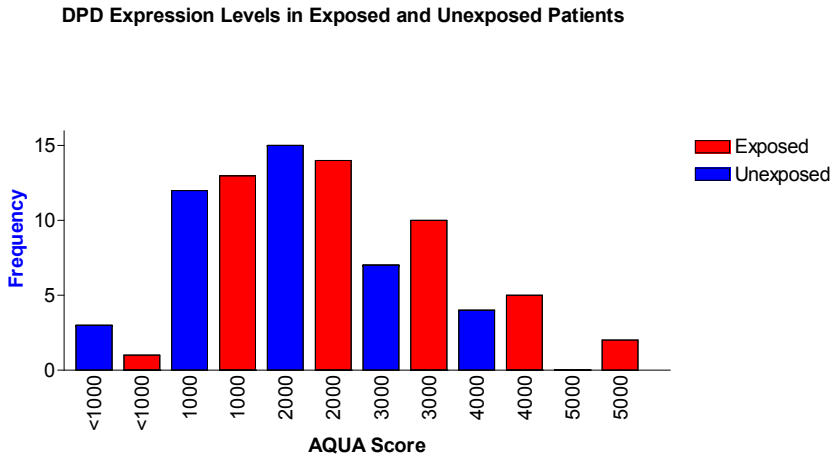


Figure A1.3 Distribution of DPD protein expression among the exposed (red) and unexposed (blue) as quantified by AQUA

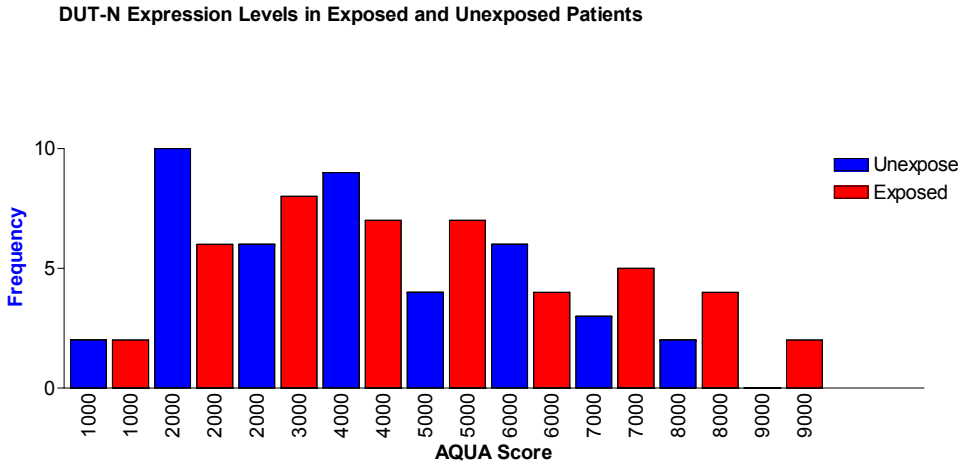
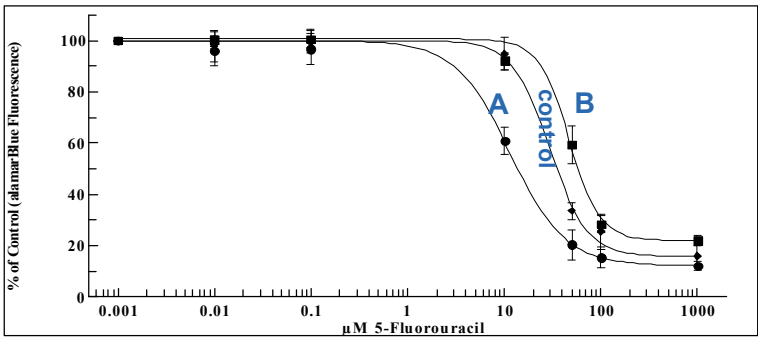


Figure A1.4 Distribution of DUT-N protein expression among the exposed (red) and unexposed (blue) as quantified by AQUA



A = sensitive
B = resistance

Figure A1.5 Example of dose-response curves used to quantify altered 5-FU phenotype

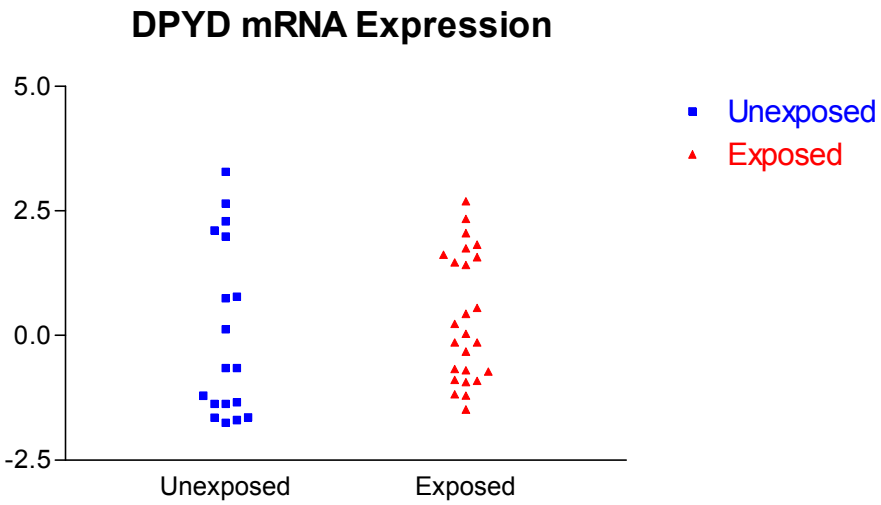


Figure A1.6 DPYD mRNA expression as determined by microarray in exposed (red) and unexposed (blue) samples

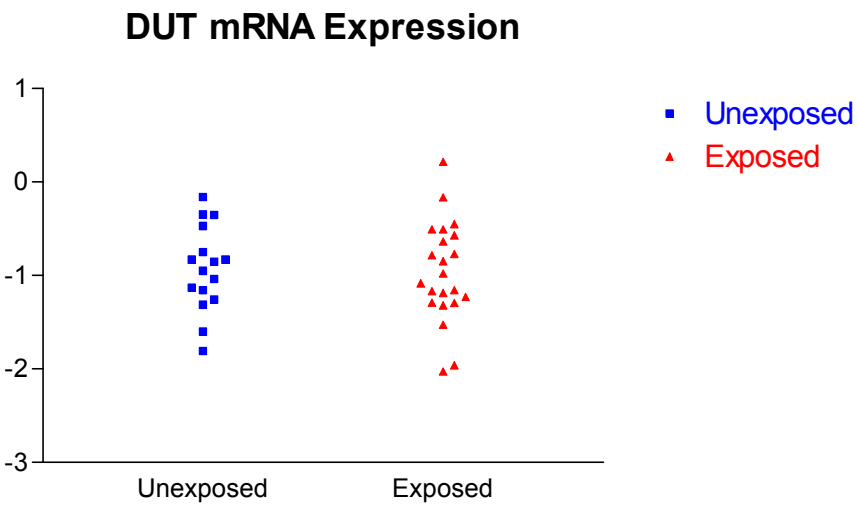


Figure A1.7 DUT mRNA expression as determined by microarray in exposed (red) and unexposed (blue) samples

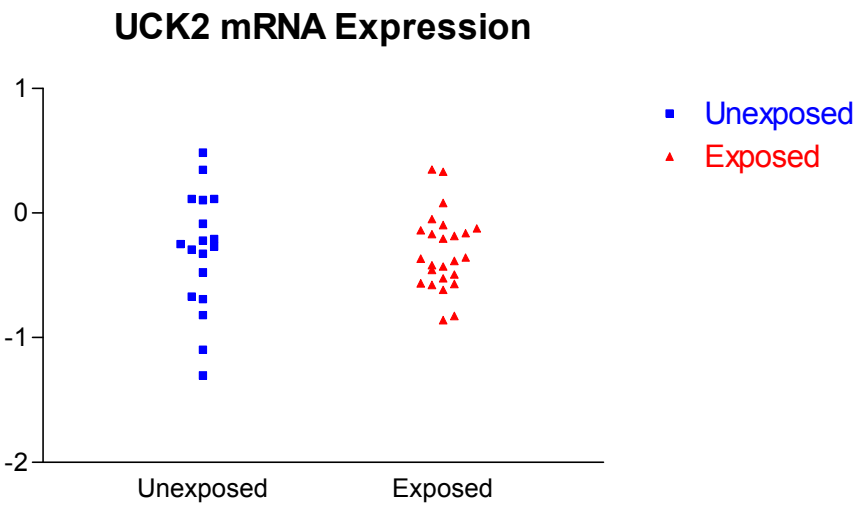


Figure A1.8 UCK2 mRNA expression as determined by microarray in exposed (red) and unexposed (blue) samples