COMPARATIVE ANALYSIS OF EPIGENETIC AND GENE EXPRESSION ENDPOINTS
BETWEEN TUMOROUS AND NON-TUMOROUS TISSUES FROM HCV-POSITIVE
PATIENTS WITH HEPATOCELLULAR CARCINOMA

Eric John Formeister

A thesis submitted to the faculty of the University of North Carolina at
Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in
the Gillings School of Global Public Health, Department of Environmental Sciences and
Engineering

Chapel Hill
2010

Approved by:

Dr. Ivan I. Rusyn, M.D., Ph.D

Dr. Igor I. Pogribny, M.D., Ph.D

Dr. Melissa A. Troester, Ph.D, M.P.H.

ABSTRACT

ERIC JOHN FORMEISTER: Comparative analysis of epigenetic and gene expression
endpoints between tumorous and non-tumorous tissues from HCV-positive patients with
hepatocellular carcinoma
(Under the direction of Ivan I. Rusyn)

Transcriptional silencing induced by promoter CpG island hypermethylation is an

important epigenetic mechanism of hepatocarcinogenesis. The goals of our study were to

examine promoter methylation and mRNA levels of candidate genes, as well as global

changes in DNA methylation, in a cohort of HCV-positive HCC patients from Japan.

Methylation-specific PCR was used to assess the methylation status of seven cancer-related

genes, while the methylation status of long interspersed nuclear elements was used as marker

of global genomic methylation, in tissues obtained from patients who underwent tumor

resection surgery. Methylation frequencies for most of the genes were significantly higher in

tumorous versus non-tumorous tissues. The methylation status of only three genes correlated

with reduced mRNA levels. Genomic DNA was significantly more hypomethylated in

tumorous tissues, and was associated with shorter recurrence but not with clinicopathological

variables. In summary, this study establishes an aberrant gene-specific and global

methylation profile in HCV-associated HCCs.

ACKNOWLEDGMENTS

I wish to acknowledge several people who have motivated, inspired, and helped me throughout my graduate education at the University of North Carolina. I am indebted to Dr. Masato Tsuchiya for allowing me to conduct research with tissue samples from his patients and for his generous, patient guidance throughout my research project. I am grateful to Dr. Igor Pogribny and his laboratory colleagues at the National Center for Toxicological Research for sharing research expertise in the epigenetic mechanisms of carcinogenesis, and to Dr. Melissa Troester for her help in preparing and defending this master's thesis. I am especially thankful for Dr. Ivan Rusyn's support and mentorship throughout my program and for all of the members of the Rusyn Laboratory for making the last two years an exciting academic endeavor. I wish to thank Dr. Scott Magness for piquing my curiosity for biomedical research and for imparting on me some of his enthusiasm and passion for academic research. Finally, I would like to acknowledge the gracious departmental and graduate school financial support I have received for my graduate study at UNC.

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ABBREVIATIONS

APC   adenomatous polyposis coli gene

GSTP1   glutathione S-transferase π 1 gene

HCC   hepatocellular carcinoma

LINE-1   long interspersed nuclear element-1

MGMT O^6-Methylguanine-DNA methyltransferase gene

MSP   methylation-specific PCR

NT   non-tumorous

P16^{INK4A} cyclin-dependent kinase inhibitor 2A gene

PCR   polymerase chain reaction

RASSF1A   Ras association domain family 1A gene

RFS   recurrence-free survival

RIZ1   retinoblastoma protein-interacting zing-finger 1 gene

SOCS-1   suppressor of cytokine signaling 1 gene

T   tumorous

TSG   tumor suppressor gene

CHAPTER 1

HEPTATOCELLULAR CARCINOMA BACKGROUND AND LITERATURE REVIEW

Epidemiology, Risk Factors, and Pathogenesis

  Hepatocellular carcinoma (HCC) is the fifth most common human neoplasm and the third most fatal worldwide (1). Over 80% of HCC cases are attributable to three principal etiological factors: chronic infection with hepatitis C virus (HCV) or hepatitis B virus (HBV) and aflatoxin B1 (AFB1) exposure (2). There is profound geographic variation in both HCC incidence and the background liver disease, with the highest density of cases occurring in Southeast Asia due to chronic HBV infection (as in China) or HCV infection (as in Japan) (3). Despite decreasing incidence trends for many major human cancers, HCC incidence has actually risen over the last three decades in several countries, including the U.S. and Japan, due to increasing prevalence of HCV infection (4 – 6). In Japan, HCV infection is the major cause of HCC and is implicated in over 70% of all HCC cases (4). Because the molecular pathogenesis of HCC is specific to the underlying liver pathology, experimental investigations of distinct HCC sub-types are needed in order to elucidate the etiology-specific genetic and epigenetic changes that facilitate liver tumorigenesis. Thus, the following thesis details the epigenetic investigations of tissue samples from a homogeneous cohort of HCV-positive, HBV-negative Japanese patients who underwent curative tumor resection surgery.

  The current multi-stage histopathologic model of HCV-associated hepatocarcinogenesis begins with chronic HCV infection, which persists in 80% of

individuals. After protracted infection with HCV, close to 30% will eventually develop liver cirrhosis, the predominant antecedent liver pathology in hepatocarcinogenesis. Virtually all (>97%) individuals who are chronically infected with HCV and develop HCC exhibit HCV-induced cirrhosis, while the rest demonstrate advanced fibrosis and/or hepatitis (7). Overall, then, HCC develops from the pathologic context of a persistent necroinflammatory hepatic disease (8). Similar to other epithelial cancers, development of HCC has a prolonged induction period lasting 20 to 40 years that includes 10 to 30 years of preneoplastic lesions, followed by 5 to 10 years of developing dysplastic hepatocytes and nodules before clinical detection of HCC (2).

Despite the well-defined histologic progression from normal liver to HCC, the molecular pathogenesis of HCV-associated HCC is largely unknown (8). Recent studies, however, have provided strong molecular evidence for the role of inflammation-mediated increases in oxidative stress and increased risk of tumor formation. Intractable HCV infection elicits a chronic inflammatory response characterized by overproduction of reactive oxygen species (ROS) (9). High ROS levels overpower the liver's mechanisms for anti-oxidant scavenging, and are either directly cytotoxic or increase the rate of oxidative DNA damage, including the formation of highly mutagenic 8-hydroxy-deoxyguanosine DNA lesion. Furthermore, it has been shown in a mouse model of HCV-associated carcinogenesis that HCV infection can directly increase the production of ROS, independently of a state of chronic inflammation (10).

A number of dysregulated molecular signaling pathways have been implicated in HCC tumorigenesis, for instance, activation of the Wnt/β-catenin pathway and inactivation of E-cadherin (9). Interestingly, the core proteins encoded in the HCV genome have been

shown to directly interact with and mediate activity of several of the cyclin/cyclin-dependent kinase cell cycle control proteins (11). Thus, the landscape of molecular alterations in HCV-associated hepatocarcinogenesis is beginning to emerge, but is far from complete. Additionally, the contribution of epigenetic changes to the aberrant molecular milieu in preneoplastic and neoplastic liver tissue is incompletely characterized.

Clinical Management and Tumor Recurrence

Clinical management of HCC is particularly challenging due to the lack of predictive, diagnostic or prognostic biomarkers and because HCC is often refractory to chemotherapy and radiation treatment (12). Common therapies in early-stage HCC include tumor resection, liver transplantation, and targeted ablation by radiofrequency or ethanol injection (13). Tumor resection is the most widely applicable curative treatment option (14); however, prognosis following tumor resection is poor, with a 5-year survival as low as 35% (12).

A major contributor to the dismal prognosis following tumor resection is the high 5-year HCC recurrence rate (75% to 100%) (14). Additional tumors in the liver remnant can develop from either intrahepatic metastasis from the primary tumor or multicentric occurrence (14). The latter suggests that genetically and epigenetically distinct tumors can arise independently from the same diseased background liver, a theory first described histopathologically by Slaughter et al. in 1953 as "field cancerization." This concept posited that additional tumors arise from histopathologically and biologically altered patches of preneoplastic tissues, and can at least partially account for the high rate of recurrence in some cancers (15). From a genetic perspective, field cancerization has been demonstrated in several cancers, including those of the head and neck, lung, skin, breast, colon and bladder

(16). Propagation of genetic mutations acquired by a stem cell produce clonal patches of genetically altered cells, which develop into larger fields of genetically compromised cells. Additional mutations and eventual clonal divergence can lead to multiple primary tumors from the same field (16). Despite the obvious clinical implications of field cancerization for risk of HCC recurrence, field cancerization has not been fully characterized in hepatocellular carcinoma, nor has field cancerization been examined through an epigenetic lens. And finally, though the underlying assumption in field cancerization is that tumor recurrences represent additional monoclonal tumors (a so-called "second primary tumor"), the literature regarding tumor clonality (e.g., monoclonal versus polyclonal) has not been consistent

Though clinicopathological features, such as tumor size, number, and differentiation, are useful in identifying patients who are at-risk for recurrence, these characteristics are seldom able to prospectively predict recurrence-free survival, partially because HCC is being diagnosed at increasingly earlier stages (17). Thus, the search for better, non-invasive, predictive biomarkers remains a high clinical priority.

Gene Expression, Epigenetics and Biomarkers

Because HCC is a cancer of heterogeneous etiologies, intensive research has focused on the applicability of gene expression profiles for molecular sub-typing of HCC cases. Several groups have used whole genome microarrays and a transcriptomic approach for identifying specific molecular classes of HCC (18 – 19). Furthermore, several investigators, including those from our laboratory, have succeeded in establishing predictive recurrence-free survival signatures based on gene expression in tumorous and non-tumorous tissues from patients undergoing curative resection, demonstrating the prognostic utility of molecular

biomarkers in investigations of HCC recurrence. However, the clinical adaptability of gene expression studies of recurrence-free survival are limited in that the process requires tissue biopsies, is resource-, time-, and data-intensive, and gene expression changes are typically quite unstable and dynamic characteristics of diseased tissues.

Due to the above limitations and the continued paucity of strong, independent predictors of recurrence-free survival, very recently, investigations into the epigenetic changes occurring during hepatocarcinogenesis have become a focus of HCC research. In contrast to genetic changes, which largely refer to alterations in the actual sequence of DNA, epigenetics refers to heritable changes in gene expression that do not involve changes in the DNA sequence (20). A well-investigated epigenetic mechanism affecting gene expression is the addition of methyl residues to cytosine nucleotides that are 5' to guanosine nucleotides in the DNA sequence (called CpG dinucleotides). CpG islands are short stretches of DNA 500 to 4,000 bp in length that are rich in CpG sequences and are found in the promoter region and first exon of more than half of all genes in the mammalian genome (21). In normal mammalian cells, CpG islands are typically unmethylated. (20).

In cancer cells, however, promoter hypermethylation results in transcriptional repression of critical tumor suppressor and other cancer-related genes and is important epigenetic event in both the initiation and progression phases of carcinogenesis (21), including hepatocarcinogenesis   (22 – 23). The observation that a myriad of tumor suppressor genes are aberrantly methylated in cancer has led to the characterization of some tumors as demonstrating a CpG island methylator phenotype (CIMP), a phenotype which identifies neoplasms with a high degree of epigenetic instability (24). Additionally, in liver and other cancers, gene-specific promoter hypermethylation is often accompanied by global

genomic hypomethylation, an epigenetic event that can lead to oncogene activation and overall genomic instability, further disposing preneoplastic tissues to malignant transformation (25).

Several groups have focused on establishing profiles of aberrant DNA methylation in both the tumorous and non-tumorous tissue from the surgical margin in order to characterize epigenetically regulated genes that are important in hepatocarcinogenesis and might serve as useful clinical biomarkers. Among the most frequently cited cancer-related genes found to be hypermethylated in tumorous tissues are $P16^{INK4A}$, *RASSF1A*, *SOCS-1*, *GSTP1, APC, RIZ1,* and *MGMT* (22; 26 – 31). Methylation profiling of HCC tissues has largely followed a paradigm of trying to map the progressive accumulation of aberrant methylation of specific tumor suppressor genes throughout the histopathologic steps of hepatocarcinogenesis (22; 32 – 33). Apart from cementing the notion that cancer is a multi-hit genetic and epigenetic disease, these studies have been crucially important in providing evidence for field cancerization of HCC, specifically, high frequencies of methylation in the surrounding non-neoplastic tissue.

Others have tested the potential utility of using methylation status as a predictor of either overall or recurrence-free survival after resection, and found that methylation of *GSTP1, CDH1, P16^{INK4A}, CRABP1*, and *SYK* in tumorous tissues corresponds to shortened overall survival (22; 34 – 35), while specific promoter hypermethylation of *MGMT* in tumorous tissues and *RIZ1* in the non-tumorous surgical margin are significantly associated with recurrence-free survival (29). In addition to findings that gene-specific methylation status is associated with recurrence-free survival, several investigators have demonstrated that global DNA hypomethylation in tumorous tissues is significantly inversely correlated

with overall patient survival (25; 36). Finally, the recent finding that aberrant methylation is detectable in serum DNA in patients several years before clinical diagnosis of HCC (37) offers the exciting potential for the clinical application of non-invasive methylation analysis for predict HCC cases or prognosticating recurrence-free survival after tumor resection.

Scope of the Thesis and Outline of Findings

Almost all of the aforementioned epigenetic studies of HCC consisted of patients who were heterogeneous with respect to the underlying disease etiology, and thus somewhat hinder the capacity to delineate the epigenetic aspects of specific HCC sub-types. Thus, characterization of epigenetic changes specific to disease etiology and identification of genes whose promoter methylation status might be predictive of recurrence-free survival are still important clinical goals. The research detailed herein consisted of a well-defined cohort of HCV-positive, HBV-negative Japanese HCC patients who underwent curative tumor resection surgery and who were followed thereafter to determine the time to tumor recurrence. This report describes both differential hypermethylation of seven cancer-related genes and differential global genomic hypomethylation between tumorous and adjacent non-tumorous tissues. I have identified associations between the methylation status of several tumor suppressor genes and clinicopathological features and show that methylation of *RIZ1* in non-tumorous tissues is a significant predictor of earlier tumor recurrence. Additionally, I demonstrate that the level of global genomic hypomethylation in tumorous tissues is significantly higher in patients with shorter versus longer recurrence-free survival times, and that the level of hypomethylation in non-tumorous tissues from all patients is associated with the time to recurrence. Comparative analyses between the methylation status of the cancer-

related genes and corresponding gene expression data are shown. Finally, I describe efforts to use a predictive recurrence-free survival gene expression signature, derived from the same patients, to guide the search for new genes that are potentially regulated by promoter hypermethylation during carcinogenesis. Overall, it is clear that epigenetic changes in both tumorous and non-tumorous tissue from the surgical margin can yield prognosis-related information and can supplement and enhance typical clinicopathological data. Clinically, examination of the epigenetic changes in both the resected tumor and the liver remnant can provide important complementary information to be used in post-operative management.

CHAPTER 2

MATERIALS AND METHODS

Patient Population and Sample Collection

The patient cohort consisted of 49 HCV-positive, HBV-negative patients with primary HCCs who underwent curative resection surgery at the University of Yamanashi Hospital (Yamanashi Prefecture, Japan) between 2000 and 2007 (mean age = 66.2±8.1 years; 37 males and 12 females). The viral hepatitis status was determined by one or more of the following techniques: (i) presence of anti-HCV and anti-HBV reactive serum proteins; (ii) reverse transcription-PCR for serum HCV-RNA; or (iii) branched DNA-HCV probe assay. After surgery, patients returned each month to the ambulatory care clinic for follow-up tests, including measurement of α-fetoprotein levels. Ultrasounds or computed tomography scans of the liver were performed every 3 or 6 months, respectively, to determine the time of recurrence. Follow-up data was collected until a detectable recurrence, patient death, or the end of the study period (July, 2008). Informed consent was obtained in accordance with the Institutional Board on Ethics for Human Science at the University of Yamanashi.

Following removal of the tumor(s), fresh tissue samples were collected from the tumorous and the non-tumorous surgical margin, frozen in liquid nitrogen and stored at -80º C until laboratory processing. Due to insufficient tissue quantities from several of the patients, 43 tumorous and 45 non-tumorous tissue samples were used in the present study, representing 39 paired tissue samples from the same patients. DNA from non-transplant

grade human livers (free from liver disease according to the pathology reports) was kindly

provided by Drs. Stephen Ferguson and Jonathan Jackson (LifeTechnologies/CellzDirect,

Durham, NC) and used as controls. Figure 1 offers an overview of the experimental setup.

Figure 1. Schematic of experimental protocol. DNA was isolated from tissue samples from resected tumors and the corresponding non-tumorous surgical margin. Control liver DNA was obtained from livers without pathology and all DNA samples were subjected to bisulfite conversion for use in methylation-specific PCR (MSP) for specific genes or for use in PCR amplification of LINE-1/HinfI digestion. Survival and correlational analyses were performed to investigate the relationships between promoter methylation status or global hypomethylation levels and clinicopathologic variables and recurrence-free survival. Microarray data from the same tumorous and non-tumorous tissue were used to compare gene expression to promoter methylation status for the genes of interest, as well as to identify new candidate genes for which MSP analyses were performed.

DNA isolation and sodium bisulfite conversion

DNA was isolated from frozen tissue samples by a procedure that was slightly

modified from one reported previously (38). Briefly, ~100 mg tissue was thawed and

suspended in 1 mL 1X PBS (Sigma, St. Louis, MO), then mechanically homogenized with a

Retsch Tissue Lyser (Qiagen, Valencia, CA). The homogenate was centrifuged at 1,700 x g for 5 minutes to pellet the nuclei. The nuclei were re-suspended and digested in 1 mL Cell Lysis Solution (5 Prime Inc., Gaithersburg, MD) and 25 µL Proteinase K (Roche Diagnostics, Indianapolis, IN) overnight at 4º C on an orbital shaker. Protein was precipitated using 330 µL protein precipitation solution (5 Prime Inc., Gaithersburg, MD) and centrifugation at 2,000 x g for 10 minutes. DNA/RNA was precipitated from the supernatant using 100% isopropanol, washed with 70% ethanol, then re-suspended in 1 mL Cell Lysis Solution for 2 hours at 4º C on an orbital shaker. Following RNA digestion with 4.5 µL Ribonuclease A (Sigma, St. Louis, MO) at 37º C for 30 minutes, the above protocol for protein precipitation and DNA precipitation and washing was repeated. DNA was re-suspended in 150 µL ddH$_2$O and stored at -80º C until use.

In order to determine the specific promoter methylation status of several cancer-related genes in tumorous, non-tumorous, and control liver samples, isolated DNA was subjected to a protocol that uses sodium bisulfite treatment to chemically convert unmethylated cytosine residues to uracil, while methylated cytosines (5-methylcytosine) remain unchanged (38). This allows the design of two different primer sets, one which will specifically amplify methylated DNA and one which will specifically amplify unmethylated DNA. In these experiments, a commercially available kit for rapid sodium bisulfite conversion was used (EZ DNA Methylation-Gold Kit, Zymo Research, Orange, CA). For bisulfite conversion, 2 µg of isolated genomic DNA was used following the manufacturer's suggested protocol.

Methylation-specific Polymerase Chain Reaction (MSP)

Methylation-specific polymerase chain reaction (MSP) takes advantage of the chemical conversion of unmethylated cytosine residues to uracil following sodium bisulfite treatment (39) and the selective amplification by the methylated or unmethylated primers. Each bisulfite-treated DNA sample was run in two reactions; one with a methylated primer set and one with an unmethylated primer set. The methylated and unmethylated primer sequences for each of the seven cancer-related genes were taken from previously published reports. The sequences, amplicon product sizes and associated references for $P16^{INK4A}$, *SOCS-1*, *RASSF1A*, *APC*, *GSTP1*, *RIZ1* and *MGMT* are shown in Table 1.

Table 1. Cancer-related genes, associated functions, and primer sequences used in MSP analyses. Primer sequences were from previous publications (see Ref.) and read from 5' → 3'. Separate reactions were carried out using unmethylation-specific primers and methylation-specific primers for each gene and each bisulfite-trated DNA sample.

Gene	Name/Function	Forward sequence	Reverse Sequence	Product Size	Ref.
p16	p16INK4A/cell cycle regulator and mitotic checkpoint (CDK inhibitor)	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	151 bp	26
		M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	150 bp	
SOCS-1	Suppressor of cytokine signaling 1/Inhibitor of the Jak/Stat pathway	U: TTATGAGTATTTGTGTGTATTTTTAGGTTGGTT	U: CACTAACAACAAACTCCTACAACAACCA	175 bp	28
		M: TTC GCG TGT ATT TTT AGG TCG GTC	M: CGACACAACTCCTACAACGA CG	160 bp	
RASSF1A	Ras-effector homologue 1A/Inhibitor of the EGFR/Ras pathway	U: TTTGGTTGGAGTGTGTTAATGTG	U: CAAACCCCACAAACTAAAAACAA	105 bp	27
		M: GTGTTAACGCGTTGCGTATC	M: AACCCCGCGAACTAAAAACGA	93 bp	
APC	Adenomatous polyposis coli/Inhibitor of Wnt/β-catenin signaling	U: GTGTTTTATTGTGGAGTGTGGGTT	U: CCAATCAACAAACTCCCAACAA	108 bp	22
		M: TATTGCGGAGTGCGGGTC	M: TCGACGAACTCCCGACGA	98 bp	
GSTP1	Glutathione S-transferase π1/Phase II xenobiotic metabolism	U: GATGTTTGGGGTGTAGTGGTTGTT	U: CCACCCCAATACTAAATCACAACA	97 bp	22
		M: TTCGGGGTGTAGCGGTCGTC	M: GCCCCAATACTAAATCACGACG	91 bp	
RIZ1	Retinoblastoma protein-interacting zing-finger/ inhibitor of cell growth	U: TGGTGGTTATTGGGTGATGGT	U: ACTATTTCACCAACCCCAAGA	175 bp	29
		M: GTGGTGGTTATTGGGCGACGGC	M: GCTATTTCGCCGACCCCGACG	176 bp	
MGMT	O⁶-Methylguanine-DNA methyltransferase/DNA repair	U: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	U: AACTCCACACTCTTCCAAAAACAAAACA	93 bp	31
		M: TTTCGACGTTCGTAGGTTTTCGC	M: GCACTCTTCCGAAAACGAAACG	81 bp	

U, unmethylation-specific primer; M, methylation-specific primer

Briefly, 50 ng of bisulfite-converted DNA (10 µL), 50 pmol forward and reverse primers (5 µL each, Nucleic Acids Core Facility, UNC-Chapel Hill), 5 µL ddH$_2$O, and 25 µL 2X Amplitaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA), for a final reaction volume of 50 µL, were subjected to MSP under the following conditions: 95º C for 10 minutes, 35 cycles of 45 seconds at 95º C, 60 seconds at the annealing temperature specific for each primer set, 60 seconds at 72º C, and a final 10 minute extension at 72º C. The final PCR products were vacuum-concentrated to 20 µL and run on a 1.75% agarose gel. Ethidium bromide stain and Kodak ID Imager/Image Analysis Software (Sigma, St. Louis, MO) were used for DNA visualization and image processing. The presence of a band at the expected fragment length was scored as positive for methylation; absence was scored as negative. Figure 2 shows a representative MSP gel image of *RIZ1*, with all of the tumorous DNA methylated and most of the non-tumorous DNA unmethylated.

Figure 2. Representative picture of MSP products on 1.75% agarose gel. MSP reaction conditions were as described in the methods. Separate MSP reactions were performed for *RIZ1* for DNA samples from each patient, one with methylated primers (top, expected fragment length 176 bp) and one with unmethylated primers (bottom, expected fragment length 175 bp). Methylated and unmethylated primers will selectively amplify DNA that is methylated or unmethylated, respectively. Patient numbering is arbitrary. U, unmethylated control human DNA; M, methylated control human DNA; T, tumorous tissue; NT, non-tumorous tissue from the surgical margin. DNA ladder is shown on the left.

Combined Bisulfite Restriction Analysis (COBRA) of LINE-1

There are about half a million long interspersed nuclear elements (LINE) in the human genome and DNA methylation occurs mainly in these repetitive elements. Thus, examining LINE-1 methylation is a suitable proxy for evaluating global genomic methylation (40). To assess the level of global genomic methylation, a 413 bp region of LINE-1 was amplified via MSP as previously described (40). The product was aliquoted into two samples; half was digested with HinfI restriction endonuclease (New England Biolabs, Ipswich, MA) and half was left undigested. Each treatment was run in tandem on a 1.75% agarose gel and visualized as detailed above. Because Hinf1 will only digest repetitive elements that were originally methylated, the relative level of hypomethylation can be quantified using densitometry of the band intensities with the aforementioned imager and image software. Specifically, a higher ratio of the digested to undigested 413 bp band intensity indicates that less of the DNA from that sample is methylated, that is, DNA is more globally *hypo*methylated in that tissue sample. Figure 3 shows a representative gel image used to quantitate relative band intensities, with more of the non-tumorous DNA originally methylated than the tumorous DNA.
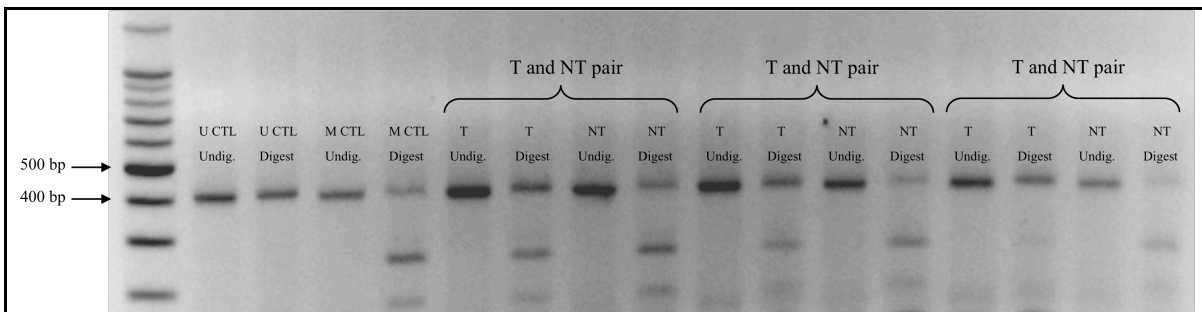
Figure 3. Representative picture of LINE-1 PCR products on 1.75% agarose gel. A 413 bp region of LINE-1 was amplified according to the reaction conditions described in the methods. PCR products were divided into two aliquots; one was left undigested while one was digested with the HinfI restriction enzyme, which will only cut LINE-1 sequences that are methylated. The ratio of the 413 bp band intensity in the digested aliquot to the 413 bp band intensity in the undigested aliquot were quantitated using densitometry, yielding an index of relative hypomethylation of LINE-1 in the tissue sample. Thus, 413 bp bands of higher intensity in the digested aliquot demonstrating that this sample was more unmethylated (hypomethylated). Possible digestion products were 285 bp, 247 bp, 166 bp, 128 bp and 38 bp. Bands of 285 and 166 bp appear on this gel, but were not used in quantitating relative hypomethylation. Tumorous and non-tumorous tissue pairs from the same patient are indicated. U CTL, unmethylated control human DNA; M CTL, methylated control human DNA; T, tumorous tissues; NT, non-tumorous tissues from the surgical margin; Undig., undigested DNA sample.

Gene Expression Data

As part of a companion study conducted by other members of the Rusyn Laboratory (Tsuchiya et al., *Mol Cancer*, 2010 (in press)), gene expression data from microarrays were available for most of the tumorous (41/43) and non-tumorous (43/45) samples. Raw microarray data was archived in Gene Expression Omnibus (GSE17856) and is available to the public. Levels of mRNA were compared to methylation status of the seven genes investigated and analyses were performed to determine the relationship between gene expression, recurrence-free survival and clinicopathological variables.

Gene expression data was further used to guide the search of new candidate genes that are potentially regulated by promoter hypermethylation. The submitted study referenced above (Tsuchiya et al., *Mol Cancer*, 2010 (in press)) established a predictive recurrence-free survival signature based on gene expression of 91 genes in the non-tumorous tissue samples from patients with a late (>1 year) recurrence. Cox scores for the association between gene expression and recurrence-free survival were obtained for all genes. The ten genes with the largest positive Cox scores (associated with earlier recurrence) and the ten genes with the largest negative Cox scores (associated with later recurrence) were selected. A 2,000 nucleotide sequence of DNA obtained from the Genome Browser at the University of California, Santa Cruz (http://genome.ucsc.edu/), including 1000 bp upstream and 1000 bp downstream of the transcriptional start site, was analyzed for the presence of potential CpG islands using Methyl Primer Express Software (Version 1.0, Applied Biosystems, Foster City, CA). Unmethylated and methylated primer sets were designed using the software for those genes for which putative promoter CpG islands were detected and synthesized at the UNC Nucleic Acids Core Facility (Chapel Hill, NC). To screen for aberrant promoter

methylation of these genes, methylation status of each gene was determined using MSP on

DNA from 8 paired tumorous and non-tumorous tissues, including 4 pairs from patients with

an early (<2 years) recurrence and 4 pairs from patients with a late (>2 years) recurrence.

Statistical Analyses

Most of the statistical tests were performed separately within both the tumorous and

non-tumorous sample cohorts. McNemar's, chi-square, Fisher's exact tests and both paired

and unpaired Student's t-test were used to compare methylation frequencies between

tumorous and non-tumorous samples, to determine associations between methylation status

of each gene and clinicopathological variables, to examine differences in gene expression

according to methylation status, and to compare LINE-1 methylation between tumorous and

non-tumorous samples. Univariate and multivariate Cox proportional hazard models were

used to investigate relationships between promoter methylation status, gene expression, and

clinicopathological data with recurrence-free survival with JMP software (version 6, SAS

Institute Inc., Cary, NC). Kaplan-Meier curves were generated and Mantel-Cox log-rank tests

were performed to analyze the association between recurrence-free survival and gene-

specific or global methylation status using software from GraphPad Prism (version 5, San

Diego, CA). A p-value <0.05 was considered statistically significant. Gene expression values

($\log_2$ transformed ratios of expression between the test sample and a universal reference)

were visualized using Cluster and TreeView algorithms (41).

CHAPTER 3

RESULTS

Clinicopathological Characteristics of the Patient Population

In all of the statistical analyses performed in this study, the full patient cohort (49 subjects) was divided into patients for which DNA and gene expression data from HCC samples (n=43), or non-tumorous samples (n=45) were available. There were 39 paired tumorous and non-tumorous samples from the same patient. The patients in each sub-cohort did not differ significantly with respect to any of the clinicopathological variables (data not shown). As a first step to consider the relationship between the typical clinicopathological variables of HCC resection patients, including demographic characteristics, tumor information, laboratory results, and the recurrence-free survival, univariate Cox proportional hazards analyses were performed. In both sub-cohorts, tumor number, tumor diameter, and tumor stage were significantly associated with an earlier recurrence according to Log-Rank tests (Table 2).

Table 2. Univariate Cox proportional hazards analyses of the association between clinicopathological variables and recurrence-free survival in tumorous and non-tumorous tissues. Hazard ratios >1 represent an increase in risk for recurrence based on methylation status. Bold numbers denote p-values <0.05 (Log-Rank Test).

Clinicopathological variable	Tumorous samples (n=43)			Non-Tumorous samples (n=45)		
	Hazard Ratio	95% CI	p-value	Hazard Ratio	95% CI	p-value
Age	0.99	0.95 - 1.05	0.80	1.00	0.96 - 1.05	0.95
Sex (Male)	1.43	0.95 - 2.35	0.09	1.27	0.87 - 1.96	0.22
Tumor Diameter	1.55	1.10 - 2.11	**0.01**	1.58	1.16 - 2.08	**0.005**
Tumor Number	2.22	1.08 - 4.40	**0.03**	2.31	1.14 - 4.44	**0.02**
Tumor Stage	2.40	1.33 - 4.46	**0.004**	2.47	1.38 - 4.55	**0.002**
Tumor Differentiation (moderate - poor)	1.05	0.61 - 1.62	0.84	0.95	0.58 - 1.44	0.83
Fibrosis Score	0.86	0.60 - 1.28	0.45	1.00	0.68 - 1.52	0.99
Platelet Count	1.00	0.92 - 1.09	0.99	0.96	0.89 - 1.04	0.34
ALT	1.00	0.99 - 1.01	0.67	1.00	0.99 - 1.01	0.77
Total Bilirubin	1.26	0.47 - 2.95	0.63	1.11	0.37 - 2.82	0.84
Prothrombin Time	0.99	0.96 - 1.01	0.26	0.99	0.97 - 1.02	0.44
Indocyanine Green	1.01	0.97 - 1.05	0.71	1.01	0.97 - 1.05	0.49
α-Fetoprotein	1.00	1.00 - 1.00	0.79	1.00	1.00 - 1.00	0.82

Gene-specific Promoter Methylation Analysis in Tumorous and Non-tumorous Tissues

Aberrant promoter methylation of $P16^{INK4A}$, *SOCS-1*, *RASSF1A*, *APC*, *GSTP1*, *RIZ1*, and *MGMT* is commonly reported in epigenetic studies of HCC. Here, I examined the methylation profile of these 7 genes, which are associated with a number of dysregulated pathways during carcinogenesis, in both tumorous and non-tumorous samples from HCV-positive HCC patients (Table 3). All of the genes were almost entirely unmethylated in the DNA from control human livers. The frequencies of methylation of all genes, except *MGMT,* were significantly higher (p<0.05, Fisher's exact test) in tumorous samples as compared to control liver. In non-tumorous samples, the methylation frequencies of only *SOCS-1* and *RASSF1A* were significantly higher than those in controls.

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I applied McNemar's version of the chi-square test to compare the methylation frequency between the 39 pairs of tumorous and non-tumorous tissue samples. The frequencies of methylation of $P16^{INK4A}$, *RASSF1A*, *APC*, *GSTP1*, and *RIZ1* were significantly higher in tumorous as compared to non-tumorous tissues, while the methylation frequency of *MGMT* showed an opposite trend, and *SOCS-1* exhibited equally high methylation frequencies between the sub-cohorts (Table 3).

Table 3. Frequency of promoter methylation of cancer-related genes in tumorous, non-tumorous, and control liver DNA and association with recurrence-free survival. Associations with recurrence-free survival was tested using univariate Cox proportional hazard models; p-values are from Log-Rank Tests.

Gene	Tumorous samples (n=43)				Non-Tumorous samples (n=45)				No. CTL samples methylated (%)
	No. T samples methylated (%)	Hazard Ratio	95% CI	p-value	No. NT samples methylated (%)	Hazard Ratio	95% CI	p-value	
*p16***	34/43 (79%)	0.88	0.59 - 1.40	0.57	7/45 (16%)	0.85	0.50 - 1.32	0.50	0/10 (0%)
SOCS-1	39/43 (91%)	1.05	0.63 - 2.16	0.86	41/45 (91%)	0.60	0.35 - 1.25	0.15	0/10 (0%)
*RASSF1A**	43/43 (100%)	n/a (all methylated)			31/45 (69%)	0.96	0.68 - 1.39	0.82	0/10 (0%)
*APC***	40/43 (93%)	0.91	0.49 - 2.26	0.79	9/45 (20%)	1.41	0.88 - 2.14	0.15	1/10 (10%)
*GSTP1***	23/43 (53%)	0.80	0.56 - 1.13	0.20	2/45 (4%)	0.74	0.18 - 1.61	0.52	0/10 (0%)
*RIZ1***	33/42 (79%)	0.92	0.62 – 1.46	0.71	4/45 (9%)	2.29	1.22 - 3.83	**0.01**	0/10 (0%)
*MGMT**	0/43 (0%)	n/a (all unmethylated)			7/45 (16%)	1.17	0.44 - 2.67	0.73	0/10 (0%)

T, tumorous; NT, non-tumorous; CTL, control. *p<0.05, and **p<0.001 for McNemar's Chi-Square test between tumorous and non-tumorous tissues.

In addition to identifying differences in the degree of methylation between tumorous and non-tumorous tissues, I was interested in exploring the CpG island methylator phenotype in HCCs. Figure 4 shows the distribution of the number of samples with the specified number of methylated genes in control, non-tumorous and tumorous tissues. There was a statistically significant increase in the number of methylated genes as pathology progressed from normal to neoplastic; the average number of methylated genes (± SD) in control, non-tumorous, and tumorous samples was 0.1 (±0.3), 2.2 (±1.0), and 4.9 (±1.0), respectively (p<0.001, unpaired t-tests). Though it was clear that there is a high degree of methylation in cancer-related genes from non-tumorous tissues of the surgical margin, the significant upward shift in the number

of methylated genes in the tumorous tissues suggests involvement of the CpG island

methylator phenotype in HCV-associated liver tumors (Figure 4).

Figure 4. Number of tumorous, non-tumorous and control DNA samples with 0 - 7 genes methylated. Curves are drawn to distinguish the distribution of the number of genes methylated in tumorous, non-tumorous, and control tissues. The average number of genes methylated ($\pm$ SD) was 4.9 ($\pm$ 1.0) in tumorous tissues, 2.2 ($\pm$ 1.0) in non-tumorous tissues and 0.1 ($\pm$ 0.3) in control samples. ** $p<0.001$, 2-tailed unpaired t-test.

The nonzero number of non-tumorous samples for which promoter methylation was

detected in each gene examined, and the significantly higher methylation of *SOCS-1* and

RASSF1A relative to control liver, provide evidence for epigenetic field cancerization (29;

41) within the preneoplastic tissue adjacent to HCC. To further explore this concept, and to

investigate the tumor clonality in our tissue samples, we assessed the accordance between

methylation status in tumorous and non-tumorous tissue from the same patient for each gene.

Nomoto *et al*. (43) suggested that to characterize a tumor as monoclonal or polyclonal in origin, a comparison of the methylation status between paired tumorous and non-tumorous samples might be performed. There are four possible combinations of methylation status for each tissue pair: the gene is methylated in both the non-tumorous tissue and the corresponding tumorous tissue (T+/NT+), the gene is methylated in the tumorous, but not in non-tumorous tissue (T+/NT-), the gene is methylated in neither tissue (T-/NT-), or the gene is methylated in the non-tumorous, but not the tumorous tissue (T-/NT+). The first three combinations can be identified as accordant and have been suggested to be of monoclonal origin (29). The presumption is that the tumorous tissue represents the most highly genetically and epigenetically aberrant; thus, all three of the aforementioned combinations show the degree of epigenetic alterations in the tumorous tissues is either the same or greater than in the non-tumorous tissues. However, if the pre-cancerous tissue from the surgical margin is methylated but the tumorous tissue is not (the fourth combination), this "discordant" status suggests that the tumor has arisen from a polyclonal origin, because the field is epigenetically altered in such a way that has not been preserved in the tumorous tissue. However, this analysis does not permit definitive conclusions regarding whether or not the tumor is monoclonal; rather, it only allows one to characterize the tumor as potentially polyclonally-derived or not. When comparing each sample pair type by each specific gene (Table 4), the majority of tissues were either T+/NT- (avg. 42%) or T+/NT+ (avg. 28%) (Figure 5B). However, at least one sample pair was discordant (that is, T-/NT+) for methylation in all genes except *RASSF1A* and *APC*, indicating that these tumors were polyclonally derived and providing support for multicentric HCC tumorigenesis, as described by others (17).

Table 4. Methylation status in paired tumorous and non-tumorous tissue in the same patient. (n=39).

Gene	T- /NT- (%)	T- /NT+ (%)	T+ /NT- (%)	T+ /NT+ (%)
p16	7 (18%)	1 (3%)	25 (64%)	6 (15%)
SOCS-1	1 (3%)	3 (8%)	3 (8%)	32 (82%)
RASSF1A	0 (0%)	0 (0%)	12 (31%)	27 (69%)
APC	2 (5%)	0 (0%)	29 (74%)	8 (21%)
GSTP1	17 (44%)	1 (3%)	20 (51%)	1 (3%)
RIZ1	8 (21%)	1 (3%)	27 (69%)	3 (8%)
MGMT	33 (85%)	6 (15%)	0 (0%)	0 (0%)
Average %	**25%**	**4%**	**42%**	**28%**

T, tumorous; NT, non-tumorous; (-) indicates no methylation and (+) indicates methylation in tumorous or npn-tumorous tissues

Additionally, we analyzed accordance between the methylation status in tumorous and corresponding non-tumorous tissues for all 7 genes and found that 72% of the pairs were accordant for all genes. Of the 11 cases that were not accordant for all genes, 10 were discordant for only one gene, while one was discordant for two genes (Figure 5A). This variable clonality in HCV-associated HCC underscores the complex molecular etiology of hepatocarcinogenesis.

The different tumor clonalities suggested by the accordance analysis prompted us to examine whether or not the recurrence-free survival time differed between accordant and discordant cases. Though discordant cases (the potentially polyclonally derived tumors) had a longer average time to recurrence (29.3 months) versus the accordant cases (20.9 months), this difference was not significant (p = 0.20, data not shown). We speculate that perhaps tumors derived from polyclonal preneoplastic tissue require more time to acquire the necessary epigenetic aberrations to manifest as a tumorous mass. This finding, however, is

consistent with the notion that later tumor recurrences represent multicentric, *de novo* tumor
formation, while earlier recurrences are thought to arise from monoclonal intrahepatic
recurrences (14).

Figure 5. Accordance between promoter methylation status of 7 cancer-related genes in 39 paired tumorous (T) and non-tumorous (NT) tissues. Accordance was defined as no methylation in both tumorous and non-tumorous tissues (T-/NT-), methylation in tumorous but not non-tumorous tissues (T+/NT-), or methylation in both tumorous and non-tumorous tissues (T+/NT+). Discordance was defined as methylation in the non-tumorous but not in the tumorous tissues (T-/NT+). (A), summary of methylation status for tissue pairs. Black boxes indicate cases with promoter methylation; white boxes represent cases with no methylation. (B), percent of paired T/NT tissues with each of the four possible combinations of methylation status. Means ± SEM for the methylation status of all seven genes investigated are shown.

Association Between Promoter Methylation Status, Recurrence-free Survival, and Clinicopathological Variables

To investigate the association between methylation status of the genes and
recurrence-free survival, we performed univariate Cox proportional hazards analyses for all
genes in both the tumorous and non-tumorous cohorts. We observed that hypermethylation of
RIZ1 in non-tumorous tissues was the only gene significantly associated with a higher risk
for earlier recurrence (HR=2.29; 95% CI=1.22–3.83; p=0.01, Table 3). However, this

relationship was not significant in multivariate analysis when other significant univariate clinicopathological predictors (tumor diameter, tumor number, and tumor stage) were included (data not shown). Figure 6 shows Kaplan-Meier survival curves according to the status of *RIZ1* and *P16*^{INK4A} (a representative non-significant gene) methylation in tumorous and non-tumorous tissues.

Figure 6. Kaplan-Meier recurrence-free survival curves based on promoter hypermethylation of two cancer-related genes. Curves are shown for *RIZ1*: (A), tumorous tissues, (B) non-tumorous tissues; and *p16*: (C) tumorous tissues, (D) non-tumorous tissues. P-values from Log-Rank (Mantel-Cox) tests are shown for differences in recurrence-free survival according to methylation status.
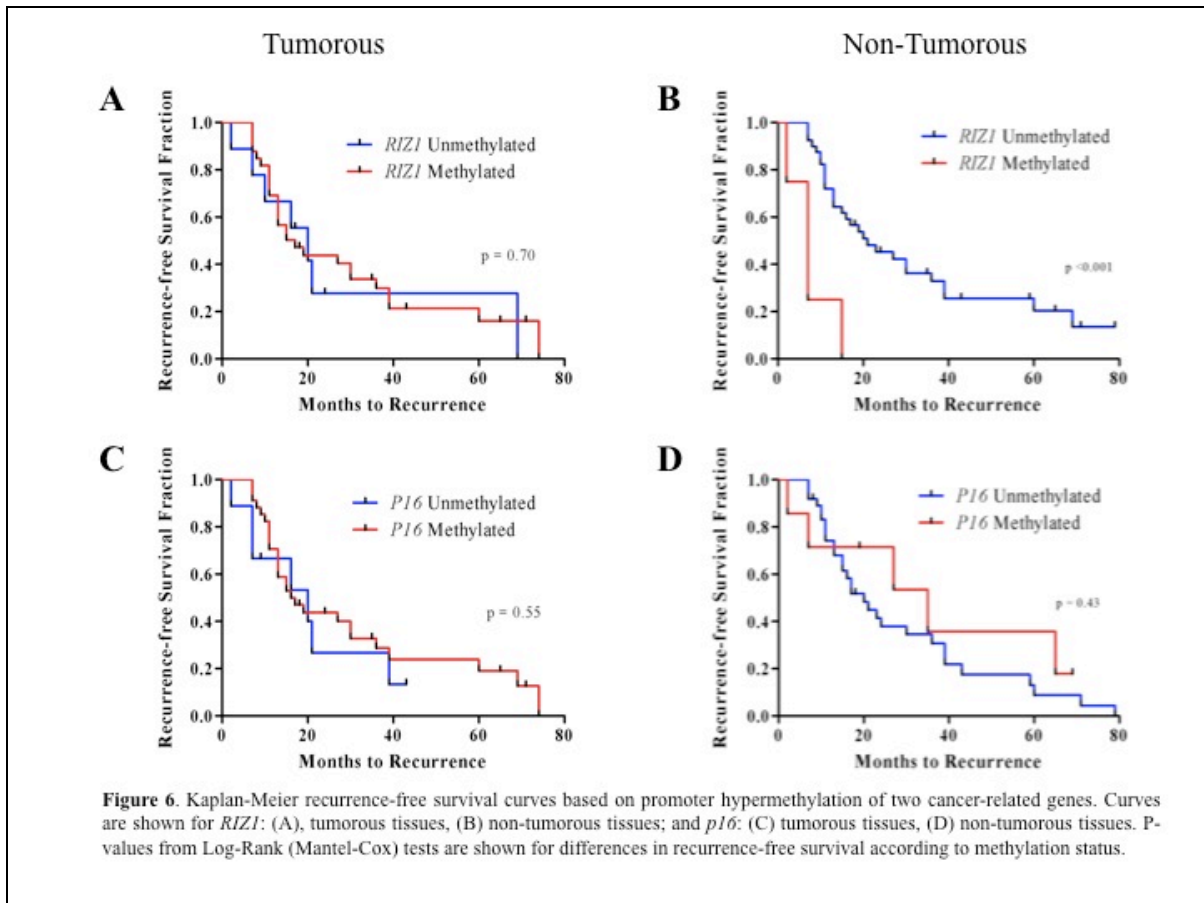
In addition, we examined the relationship between gene methylation status and clinicopathological variables. Only a few significant (p<0.05) relationships were found, including an association between *RIZ1* methylation and both age and tumor diameter in the

non-tumorous sub-cohort and an association between *GSTP1* methylation and tumor stage in the non-tumorous cohort (Table 5).

Table 5. Comparison between methylation status in tumorous and non-tumorous tissues and clinicopathological variables. Where applicable, standard deviations are shown. P-values were derived from Chi-square and Fisher's tests for categorical variables and from unpaired, two-tailed Student's t-tests for continuous variables. P-values less than 0.05 appear in bold. All of the tumorous samples showed *RASSF1A* methylation and none of the tumorous samples showed *MGMT* methylation; thus, p-values do not appear.
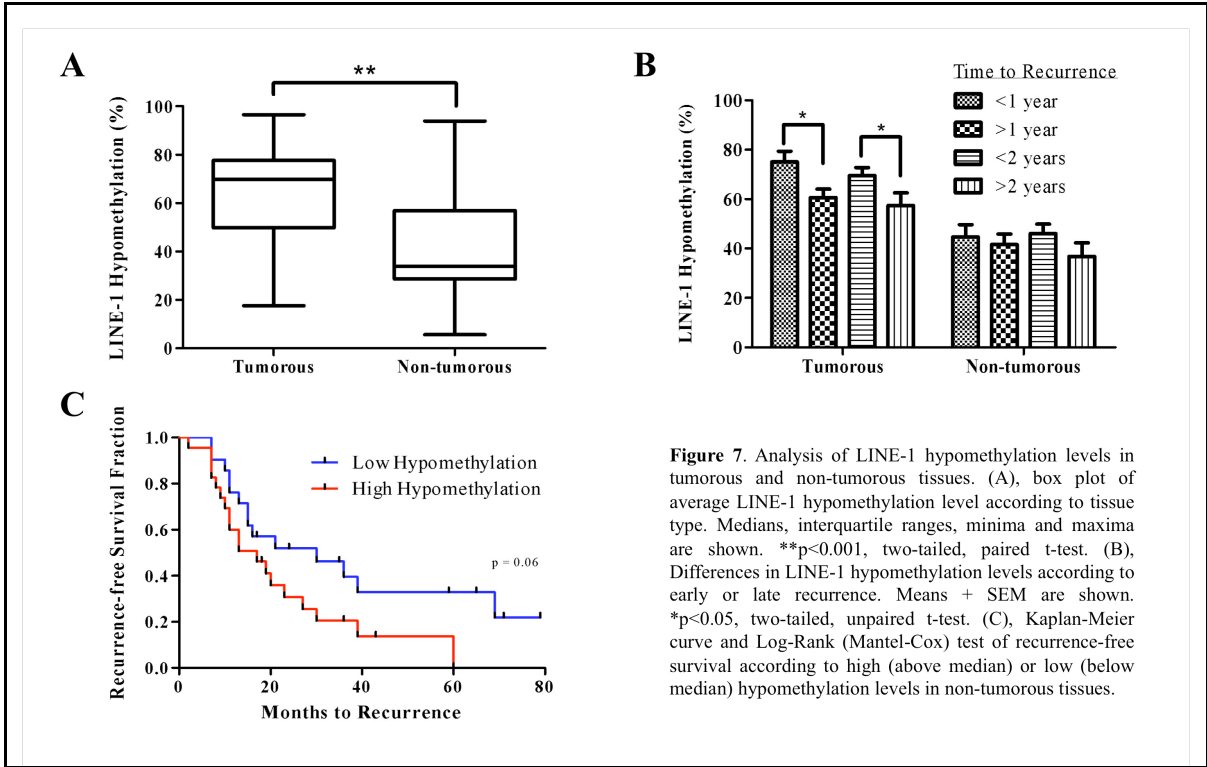
Clinicopath. Variable	No. (%) in full cohort	*P16* p-value		*SOCS-1 p-value*		*RASSF1A* p-value		*APC* p-value		*GSTP1* p-value		*RIZ1* p-value		*MGMT p-value*	
		T	NT	T	NT	T	NT	T	NT	T	NT	T	NT	T	NT
Patient No.	49	T	NT	T	NT	T	NT	T	NT	T	NT	T	NT	T	NT
Male	37 (75.5%)	0.09	1.00	1.00	1.00	n/a	0.47	1.00	0.68	1.00	0.47	0.09	0.56	n/a	0.36
Female	12 (24.5%)														
Age (yrs)	66.2 ± 8.1	0.63	0.13	0.35	0.28	n/a	0.34	0.88	0.13	0.96	0.63	0.57	**0.03**	n/a	0.16
Tumor diameter (cm)	2.9 ± 1.3	0.48	0.17	0.24	0.63	n/a	0.83	0.49	0.24	0.25	0.22	0.20	**0.02**	n/a	0.13
Single tumor	33 (67.3%)	0.23	0.41	1.00	1.00	n/a	0.17	0.54	1.00	1.00	1.00	0.23	0.06	n/a	0.36
>1 tumor	16 (32.7%)														
Stage 1	9 (18.4%)	0.50	0.51	0.51	0.49	n/a	0.58	0.40	0.76	0.94	**0.03**	0.71	0.49	n/a	0.67
Stage 2	26 (53.1%)														
Stage 3	13 (26.5%)														
Stage 4	1 (2.0%)														
Poorly or moderately differentiated	41 (83.7%)	0.32	0.09	0.84	1.00	n/a	1.00	0.93	1.00	0.96	0.33	0.71	0.56	n/a	1.00
Well differentiated	8 (16.3%)														
Fibrosis 1	3 (6.1%)	0.65	0.92	0.88	0.56	n/a	0.89	0.91	0.86	0.97	0.67	0.59	0.09	n/a	0.69
Fibrosis 2	7 (14.3%)														
Fibrosis 3	14 (28.6%)														
Fibrosis 4	25 (51.0%)														
Platelet Cnt. $(10^4/\mu L)$	12.9 ± 4.2	0.30	0.79	0.74	0.89	n/a	1.00	0.11	0.63	0.42	0.12	0.34	0.52	n/a	0.34
ALT (IU/L)	54.6 ± 38.3	0.65	0.80	0.67	0.53	n/a	0.90	0.40	0.08	0.33	0.41	0.42	0.41	n/a	0.61
Total Bilirubin (mg/dL)	0.79 ± 0.34	0.85	0.24	0.19	0.80	n/a	0.89	0.88	0.42	0.97	0.11	0.92	0.20	n/a	0.75
PT time (%)	77.7 ± 13.1	**0.04**	0.93	0.48	0.12	n/a	0.93	0.15	0.76	0.11	0.90	**0.03**	0.99	n/a	0.38
ICG (%)	18.3 ± 8.2	0.67	0.79	0.31	**0.01**	n/a	0.94	0.82	0.13	0.23	0.63	0.89	0.47	n/a	0.63
α-Fetoprotein (ng/mL)	309 ± 906	0.39	0.37	0.61	0.51	n/a	0.79	0.53	0.52	0.47	0.93	**0.04**	0.84	n/a	**0.02**

T, tumorous; NT, non-tumorous

Global DNA Methylation Analysis in Tumorous and Non-tumorous Tissues

Though promoter hypermethylation of specific genes is a common epigenetic event in hepatocarcinogenesis, this specific aberration is often concurrent with global DNA hypomethylation (44). Therefore, in addition to examining gene-specific hypermethylation, the level of global hypomethylation in tumorous and non-tumorous tissues was assessed by analyzing the level of LINE-1 methylation as a marker for global DNA methylation status. LINE-1 was significantly (p<0.001) more hypomethylated in tumorous tissues as compared to non-tumorous tissues (Figure 7A). When patients were divided into those with an earlier

recurrence (<1 year) or later recurrence (>1 year), the level of hypomethylation in the tumorous samples was significantly higher in patients with earlier recurrences. The same was true when assigning 2 years as the cutoff between early and late recurrence (Figure 7B). When patients were sub-divided into two groups based on the median value of LINE-1 hypomethylation in non-tumorous tissues, a nearly significant difference in recurrence-free survival outcome between the groups was observed; patients with hypomethylation levels above the median value experienced earlier recurrences (p=0.06, Log-Rank test (Mantel-Cox), Figure 7C). However, this relationship did not exist according to hypomethylation levels in the tumorous tissues (p = 0.61, data not shown). And when treating LINE-1 hypomethylation levels as a continuous variable, Univariate Cox proportional hazard analysis revealed that the level of global DNA hypomethylation in either tissue type was not significantly related to recurrence-free survival (p = 0.36, tumorous tissues; p = 0.26, non-tumorous tissues, data not shown). The level of LINE-1 hypomethylation in tumorous tissues was also significantly inversely correlated with ALT levels (p=0.03, Table 6). In non-tumorous tissues, LINE-1 hypomethylation was significantly directly correlated with tumor diameter (r^2 = 0.11, p = 0.03, Table 6).

Figure 7. Analysis of LINE-1 hypomethylation levels in tumorous and non-tumorous tissues. (A), box plot of average LINE-1 hypomethylation level according to tissue type. Medians, interquartile ranges, minima and maxima are shown. **p<0.001, two-tailed, paired t-test. (B), Differences in LINE-1 hypomethylation levels according to early or late recurrence. Means + SEM are shown. *p<0.05, two-tailed, unpaired t-test. (C), Kaplan-Meier curve and Log-Rank (Mantel-Cox) test of recurrence-free survival according to high (above median) or low (below median) hypomethylation levels in non-tumorous tissues.

No other significant associations between LINE-1 hypomethylation levels and clinicopathological variables were observed. Of note, there was no association between the degree of LINE-1 hypomethylation and the methylation status of any of the 7 genes, or the number of genes methylated in either sub-cohort (Table 6).

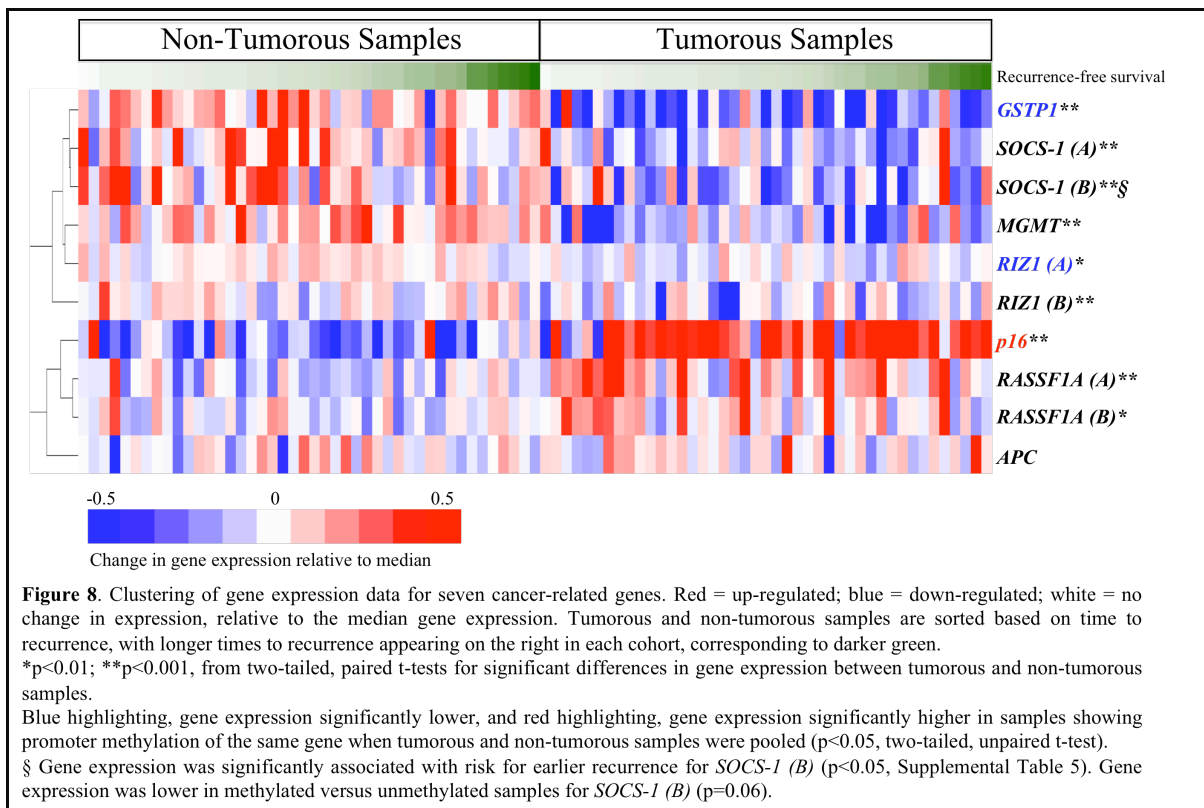| Clinicopathologic Variable	Tumorous samples (n=43)			Non-Tumorous samples (n=45)		
	No. (% or SD)	Avg. Hypomethylation (SD)	p-value	No. (% or SD)	Avg. Hypomethylation (SD)	p-value
Male	33 (76.7)	67.4 (18.5)	0.18	33 (73.3%)	44.5 (21.2)	0.38
Female	10 (23.3)	58.2 (19.6)		12 (26.7%)	37.9 (23.0)	
Age	66.4 (±8.0)		0.09	66.4 (7.9)		0.44
Diameter (cm)	2.96 (1.34)		0.61	3.01 (1.32)		**0.03**
One tumor	29 (67.4%)	65.9 (21.4)	0.75	31 (68.9%)	43.1 (22.2)	0.88
Multiple tumors	14 (32.6%)	63.9 (13.0)		14 (31.1%)	42.0 (21.1)	
Stage 1	7 (16.3%)	65.4 (29.4)	0.95	8 (17.8%)	31.3 (16.0)	0.22
Stage 2	24 (55.8%)	63.0 (21.6)		23 (51.1%)	46.7 (23.1)	
Stage 3/4	12 (27.9%)	66.7 (13.9)		14 (31.1%)	42.7 (20.8)	
Poor or moderate diff.	37 (86.0%)	66.3 (18.3)	0.49	37 (82.2%)	42.7 (20.9)	0.98
Well diff.	6 (14.0%)	58.9 (23.5)		8 (17.8%)	42.9 (26.3)	
Fibrosis 1	3 (7.0%)	41.1 (23.6)	0.14	3 (6.7%)	45.6 (22.5)	0.99
Fibrosis 2	6 (14.0%)	68.9 (19.8)		6 (13.3%)	40.6 (12.2)	
Fibrosis 3	11 (25.6%)	61.3 (17.5)		14 (31.1%)	43.1 (24.4)	
Fibrosis 4	23 (53.5%)	67.8 (21.2)		22 (48.9%)	42.7 (22.9)	
Platelet Count [10^4/uL]	12.9 (4.1)		0.18	13.1 (4.1)		0.69
ALT (IU/L)	56.1 (40.7)		**0.04**	52.1 (32.2)		0.12
Total Bilirubin [mg/dL]	0.80 (0.36)		0.14	0.76 (0.30)		0.75
Prothrombin time [%]	77.1 (13.4)		0.49	77.7 (13.0)		0.81
Indocyanine green [%]	18.5 (8.3)		0.92	18.2 (8.4)		0.96
alpha-Fetoprotein [ng/dL]	348 (962)		0.49	332 (943)		0.31
p16 (U)	9 (21%)	63.6 (27.0)	0.77	38 (84%)	42.8 (20.9)	0.94
p16 (M)	34 (79%)	65.7 (16.7)		7 (16%)	42.1 (27.3)	
SOCS-1 (U)	4 (9%)	62.0 (25.0)	0.72	4 (9%)	54.4 (29.1)	0.26
SOCS-1 (M)	39 (91%)	65.6 (20.7)		41 (91%)	41.6 (20.9)	
RASSF1A (U)	0 (0%)	n/a	n/a	14 (31%)	41.3 (21.3)	0.77
RASSF1A (M)	43 (100%)	n/a		31 (69%)	43.4 (22.1)	
APC (U)	3 (7%)	68.5 (39.2)	0.77	36 (80%)	40.6 (21.6)	0.20
APC (M)	40 (93%)	65.0 (17.5)		9 (20%)	51.1 (20.9)	
GSTP1 (U)	20 (47%)	68.2 (19.3)	0.35	43 (96%)	43.9 (21.3)	0.10
GSTP1 (M)	23 (53%)	62.7 (18.7)		2 (4%)	18.4 (18.1)	
RIZ1 (U)	9 (21%)	60.2 (22.0)	0.42	41 (91%)	43.0 (21.9)	0.78
RIZ1 (M)	33 (79%)	66.0 (18.1)		4 (9%)	39.9 (21.0)	
MGMT (U)	43 (100%)	n/a	n/a	38 (84%)	41.7 (20.4)	0.46
MGMT (M)	0 (0%)	n/a		7 (16%)	48.4 (28.8)	
No. of genes methylated	4.9 (1.0)		0.70	2.2 (1.0)		0.57

Variable Concordance between Gene Expression and Promoter Methylation Status in Tumorous and Non-tumorous Tissues

Though the functional consequence of promoter methylation is frequently presumed to be a reduction in gene expression, this relationship is seldom evaluated in clinical studies. Using microarray data collected from the same tumorous and non-tumorous samples, we were able to compare the promoter methylation status of the 7 candidate genes with their mRNA levels. There were significant differences in expression levels for 6 genes between tumorous and non-tumorous tissues (Figure 8). When tumorous and non-tumorous sub-cohorts were analyzed together, corresponding gene expression levels were significantly

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lower in samples with *RIZ1* methylation (p<0.01) and *GSTP1* methylation (p=0.01).

Interestingly, $P16^{INK4A}$ expression was significantly *higher* in methylated versus

unmethylated tissues, a relationship that was also true when analyzing only tumorous tissue.

Expression of *MGMT* was also significantly lower in samples with *MGMT* methylation in the

non-tumorous sub-cohort (Figure 8; Table 7).

Figure 8. Clustering of gene expression data for seven cancer-related genes. Red = up-regulated; blue = down-regulated; white = no change in expression, relative to the median gene expression. Tumorous and non-tumorous samples are sorted based on time to recurrence, with longer times to recurrence appearing on the right in each cohort, corresponding to darker green.
*p<0.01; **p<0.001, from two-tailed, paired t-tests for significant differences in gene expression between tumorous and non-tumorous samples.
Blue highlighting, gene expression significantly lower, and red highlighting, gene expression significantly higher in samples showing promoter methylation of the same gene when tumorous and non-tumorous samples were pooled (p<0.05, two-tailed, unpaired t-test).
§ Gene expression was significantly associated with risk for earlier recurrence for *SOCS-1 (B)* (p<0.05, Supplemental Table 5). Gene expression was lower in methylated versus unmethylated samples for *SOCS-1 (B)* (p=0.06).

The univariate Cox proportional hazards model analyses for the association between

gene expression and recurrence-free survival showed that only one probe for the *SOCS-1*

gene was significant, and only in non-tumorous tissue (HR=8.09; 95% CI=1.11–52.5;

p=0.04). For this same probe, gene expression in methylated tissues was marginally

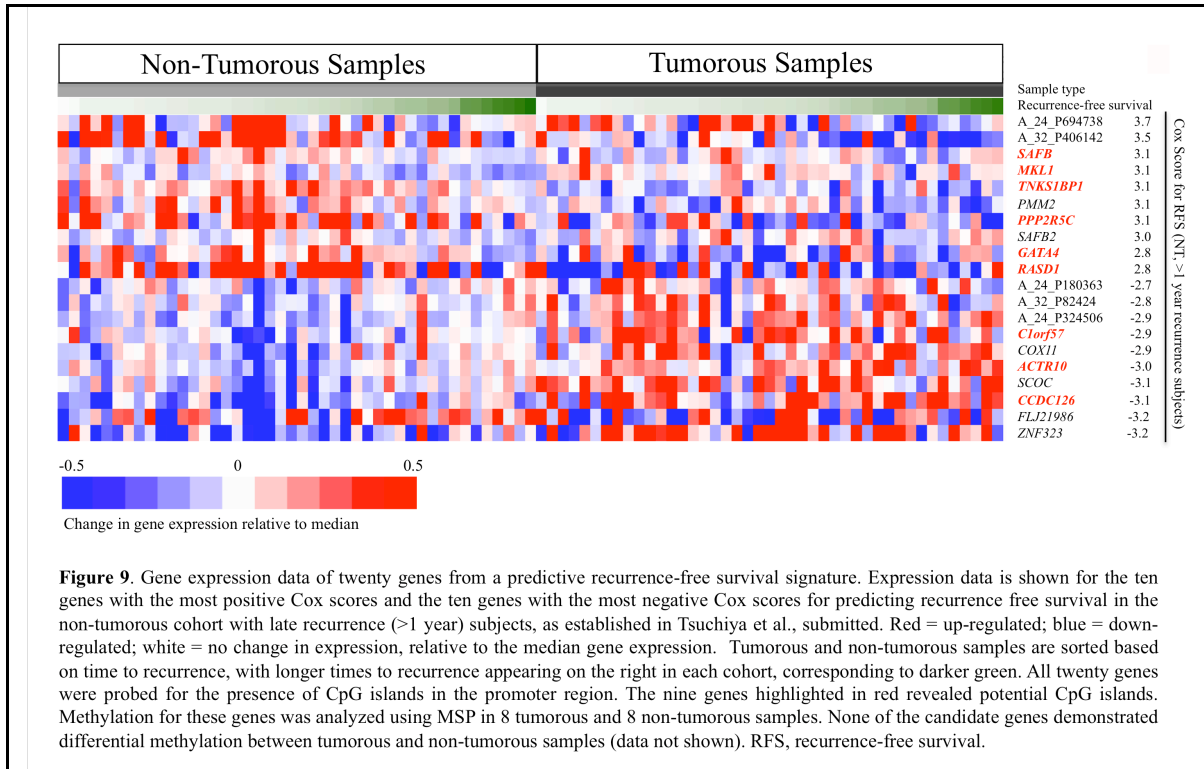significantly lower (p=0.06) in pooled tumorous and non-tumorous tissues (Table 7).

Table 7. Summary of comparative analyses between promoter methylation status, gene expression, and recurrence-free survival. Unpaired t-tests were used in comparisons of gene expression between tumorous and non-tumorous tissues and between methylated and unmethylated tissues. Univariate Cox proportional hazards models were performed to test the association between gene expression and recurrence-free survival. P-values from Log-Rank (Mantel-Cox) tests are shown for this analysis. Bold changes and values indicate significant differences. Two different probes for the same gene are distinguished by *(A)* and *(B)*.

Gene	Expression lower or higher in T vs. NT tissues? (p-value)	Expression lower or higher in M vs. U tissues? (p-value)	Expression lower or higher in M vs. U tissues? (T) (p-value)	Expression lower or higher in M vs. U tissues? (NT) (p-value)	Association between gene expression and recurrence-free survival (p-value)
P16	**Higher (<0.001)**	**Higher (<0.001)**	**Higher (0.05)**	Lower (0.74)	0.32
SOCS-1 (A)	**Lower (<0.001)**	Lower (0.37)	Lower (0.48)	Higher (0.47)	0.20
SOCS-1 (B)	**Lower (<0.001)**	Lower (0.06)	Lower (0.08)	Lower (0.22)	**0.04**
RASSF1A (A)	**Higher (<0.001)**	Higher (0.32)	n/a (all methylated)	Lower (0.18)	0.95
RASSF1A (B)	**Higher (<0.01)**	Lower (0.39)	n/a (all methylated)	Lower (0.59)	0.33
APC	Lower (0.88)	Higher (0.35)	Lower (0.50)	Higher (0.21)	0.58
GSTP1	**Lower (<0.001)**	**Lower (0.01)**	Higher (0.65)	Higher (0.48)	0.57
RIZ1 (A)	**Lower (<0.01)**	**Lower (0.008)**	Higher (0.66)	Lower (0.18)	0.82
RIZ1 (B)	**Lower (<0.001)**	Lower (0.08)	Higher (0.19)	Lower (0.62)	0.55
MGMT	**Lower (<0.001)**	Higher (0.93)	n/a (all unmethyl.)	**Lower (0.04)**	0.42

T, tumorous; NT, non-tumorous; M, methylated; U, unmethylated

We previously reported a strongly predictive recurrence-free survival signature based on gene expression data of 91 genes in non-tumorous tissue from patients with a late (>1 year) recurrence (Tsuchiya et al., *Mol Cancer*, 2010 (in press)). To examine whether differences in expression of these biomarker genes may be linked to promoter methylation, we chose the top 20 genes with the most significant Cox scores with regards to recurrence-free survival for methylation analysis (Figure 9). Of these, 9 (45%) had CpG islands in the promoter region: *SAFB*, *MKL1*, *TNKS1BP1*, *PPP2R5C*, *GATA4*, *RASD1*, *C1orf57*, *ACTR10*, and *CCDC126*. Only promoter hypermethylation of *GATA4* had been previously reported in HCC tissues (25); thus, eight of these genes represented novel genes for methylation analysis. Methylated DNA- and unmethylated DNA-specific primers were designed. We screened 8 paired tumorous and non-tumorous samples (4 from patients with <1 year

recurrence and 4 from patients with >1 year recurrence) for CpG region methylation in these

9 genes and found them to be uniformly unmethylated in both tissue types (data not shown).

Figure 9. Gene expression data of twenty genes from a predictive recurrence-free survival signature. Expression data is shown for the ten genes with the most positive Cox scores and the ten genes with the most negative Cox scores for predicting recurrence free survival in the non-tumorous cohort with late recurrence (>1 year) subjects, as established in Tsuchiya et al., submitted. Red = up-regulated; blue = down-regulated; white = no change in expression, relative to the median gene expression. Tumorous and non-tumorous samples are sorted based on time to recurrence, with longer times to recurrence appearing on the right in each cohort, corresponding to darker green. All twenty genes were probed for the presence of CpG islands in the promoter region. The nine genes highlighted in red revealed potential CpG islands. Methylation for these genes was analyzed using MSP in 8 tumorous and 8 non-tumorous samples. None of the candidate genes demonstrated differential methylation between tumorous and non-tumorous samples (data not shown). RFS, recurrence-free survival.

CHAPTER 4

DISCUSSION

Discussion of Results

Gene-specific promoter hypermethylation and global DNA hypomethylation are common epigenetic aberrations found in human liver tumors; however, answers to questions regarding the epigenetic changes specific to the underlying disease etiology remain elusive. Additionally, though the functional consequence of promoter hypermethylation is transcriptional silencing of the associated gene, this assumption often goes untested, as few have concurrently investigated both methylation and gene expression. In this study, we examined both gene-specific changes in methylation and expression levels and global DNA hypomethylation in tumorous and non-tumorous surgical margin tissues, and investigated the relationship between epigenetic changes and clinicopathological variables, recurrence-free survival and altered gene expression. Herein, we report significant differences in both specific gene hypermethylation and global genomic hypomethylation between tumorous and non-tumorous tissues and confirm the utility of *RIZ1* hypermethylation in non-tumorous tissues as a predictive biomarker of earlier recurrence following tumor resection.

Our gene-specific methylation analyses examined genes selected based on their relevance in several cancer pathways (e.g., cell cycle regulation, inhibition of the *Ras* pathway, xenobiotic metabolism and DNA repair) and previous reports of hypermethylation in HCC (22; 26 – 31). Consistent with other studies, we observed higher frequencies of

promoter methylation in tumorous versus non-tumorous tissues for $P16^{INK4A}$, *RASSF1A*, *APC*, *GSTP1*, and *RIZ1*. The high frequency of methylation in tumorous tissues for all genes, except *MGMT*, as well as the high average number of genes methylated per sample (4.9), together suggest the presence of the CpG island methylator phenotype in HCV-associated HCC, a feature that characterizes many human neoplasms (24). Interestingly, however, both *SOCS-1* and *RASSF1A* were hypermethylated at high frequencies in non-tumorous tissues and *MGMT* was significantly more methylated in non-tumorous tissues. Indeed, all of the genes examined were methylated in at least two of the non-tumorous samples, and the average number of methylated genes in non-tumorous samples was 2.2, substantiating the notion posited by others that epigenetic field defects in surrounding non-neoplastic tissues are detectable events in HCC tumorigenesis (33; 43). Others have demonstrated that promoter hypermethylation is far more frequent in non-tumorous HCV-positive liver from HCC patients as compared to non-tumorous HCV-negative liver (32), a finding that is recapitulated in the present study. Thus, the methylation profile in this etiologically distinct subclass of HCC reveals important epigenetic changes in virus-associated hepatocarcinogenesis.

As an etiologically distinct subgroup of HCC, we speculate that perhaps HCV-associated HCCs incur more epigenetic aberrations during carcinogenesis as a result of the burden of particularly high oxidative stress due to ROS overproduction. Apart from eliciting inflammation-mediated increases in cellular oxidative stress, one group has demonstrated that chronic HCV infection can increase levels of ROS independently of the inflammation response (10). Recently, Lim et al. (46) provided mechanistic evidence for the link between ROS production in HCV-infected liver and promoter hypermethylation after observing

increased promoter hypermethylation of the *E-cadherin* gene in human HCC cell lines following exposure to H_2O_2. Thus, it is reasonable to suggest that the high degree of promoter hypermethylation in both tumorous and non-tumorous tissues from HCV-associated HCC patients can at least partially be accounted for by the overwhelming levels of HCV-induced oxidative stress. That ROS production and oxidative stress is such a paramount feature of HCV-associated hepatitis and HCV-associated cirrhosis pathogenesis, studies investigating the specific link between HCV infection and epigenetic alterations in precancerous pathologies are well-advised.

In addition to demonstrating the existence of epigenetic aberrations in preneoplastic liver tissue, the high methylation frequencies observed in the surgical margin also provide epigenetic evidence for field cancerization in HCC and may be related to the rapidity of HCC recurrence following resection. Field cancerization was initially described by Slaughter *et al*. (15) when they explained that recurrent oral tumors arise from histopathologically altered fields of preneoplastic lesions. But it is now more appropriately defined as the process by which either a second primary tumor (intrahepatic metastasis) or a second field tumor (polyclonally-derived) arises from the same pre-neoplastic lesion with genetically altered cells in a distinct, biological stage (16). Previously, this model relied on the assumption that multiple liver tumors develop from a monoclonal origin (second primary tumor), an assumption that is controversial given the abundant evidence that HCCs can also include multicentric, polyclonal tumors (14). To further explore the concept of field cancerization as it relates to epigenetic changes in HCV-associated HCC, we attempted to characterize the clonality of our HCC samples by determining the accordance between the tumorous and corresponding non-tumorous tissue pairs with regard to methylation status. For all of the 7

genes examined, the majority of cases were accordant for methylation status and only a small percentage (range, 0 to 15%) were discordant (e.g., unmethylated in the tumorous tissue, while methylated in non-tumorous tissue). However, when the methylation status of all genes is considered, 11 cases (28%) were discordant for at least one gene. This result largely supports a monoclonal theory of field cancerization in HCC, but also shows that, in a minority of cases, HCV-associated HCC tumors may also arise from multicentric origins, similar to what other investigators have found (29).

A major focus of our study was to elucidate the relationship between changes in global or gene-specific DNA methylation and recurrence-free survival. The potential relevance of using methylation status as a predictor for overall or recurrence-free survival has been explored by several investigators with encouraging results. Hypermethylation of *GSTP1* and *P16^INK4A* was significantly associated with reduced overall survival (22; 33), while *MGMT* hypermethylation in tumorous tissues and *RIZ1* hypermethylation in non-tumorous tissues was significantly related to earlier recurrence (29). Our study found that only methylation of *RIZ1* in non-tumorous tissues was associated with an increased risk for earlier recurrence. This is consistent with the finding that *RIZ1* promoter hypermethylation is an early event in hepatocarcinogenesis (45) and we posit that *RIZ1* hypermethylation tested in biopsy specimens may serve as a pre-clinical marker of liver tumor development.

Promoter hypermethylation of several cancer-related genes, including those tested herein, has been found to be inversely correlated with overall survival in HCC patients (25). However, when recurrence-free survival is used as an outcome, the relationship is less certain. Our study did not find that methylation status of the candidate genes, with the exception of *RIZ1* in non-tumorous tissue, was predictive of tumor recurrence. This

observation is similar to that reported by Lou *et al*. (29) and exposes the potential weakness of using gene methylation status for predicting the clinical outcomes in HCC. However, the fact that *RIZI* methylation in non-tumorous samples is predictive of recurrence-free survival in two independent cohorts (both one that is homogeneous with respect to HCV status, and a heterogeneous one (29)) maintains our optimistic outlook for prospective use of promoter hypermethylation as a potentially useful clinical biomarker.

Though other investigators have found significant inverse correlations between the number of genes hypermethylated and patient survival, our study revealed no relationship between the number of genes methylated in either tumorous or non-tumorous sub-cohorts and reduced recurrence-free survival times. Promoter methylation status of the seven genes tested was unable to characterize post-resection prognoses in patients. We caution, however, that the number of genes examined in our study was limited and perhaps the addition of other candidate genes may yield better correlations between the number of methylated genes and recurrence-free survival. Furthermore, because dozens of aberrantly methylated genes have been identified in HCC (e.g., 25), the selection of only seven genes for methylation analysis could have perhaps stochastically missed many other genes whose methylation status might have been related to recurrence-free survival. Importantly, however, the varying results between this study and others demonstrate how specific epigenetic changes in HCC may be representative of the distinct underlying etiology.

Several groups have recently demonstrated that global DNA hypomethylation often accompanies specific gene hypermethylation in HCC and contributes to carcinogenesis via protooncogene activation and overall genomic instability (25; 35; 44). We also show that significant genomic hypomethylation, as assessed by LINE-1 methylation status, occurs

concomitantly with specific gene hypermethylation in tumorous tissues. Though we did not find a significant association between LINE-1 hypomethylation levels and recurrence-free survival using univariate Cox proportional hazards models, the level of hypomethylation in tumorous tissues from patients with earlier recurrences was significantly different than that in subjects with later recurrences, both when earlier recurrence was defined as <1 year and <2 years. Hypomethylation in non-tumorous tissues did not differ after dichotomization according to recurrence time, in agreement with the study conducted by Calvisi *et al*. (25). It is noteworthy, however, when recurrence-free survival was assessed after dividing non-tumorous tissues according to hypomethylation below and above the median level in non-tumorous tissues, a high level of hypomethylation conferred an increased risk for earlier recurrence with modest significance. This finding suggests a role for global hypomethylation in promoting the development of additional tumors from non-neoplastic surrounding tissue and is one of a myriad of results from the present study that support the notion of pervasive epigenetic alterations in the liver remnant. As an epigenetic aberration, global changes in genomic methylation appears to occur independently of gene-specific promoter hypermethylation, as the two were neither correlated, nor was the level of hypomethylation associated with the number of genes methylated in a sample.

The theme that has emerged from the epigenetic investigations of the present study, then, is that perhaps epigenetic endpoints assessed in the precancerous liver remnant following tumor resection is as informative, if not *more* informative, than an investigation of epigenetic aberrations in tumorous tissues. Only the methylations status of *RIZ1* in non-tumorous tissue samples was significantly associated with recurrence-free survival. Additionally, the methylation status of *RIZ1* and *GSTP1* in non-tumorous tissues were

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significantly associated with larger tumor diameter and more aggressive tumor stage, respectively, while methylation status for all of the genes in tumorous tissues were not related to the clinicopathological features most predictive of earlier recurrences (e.g., tumor diameter, tumor stage, and tumor number). And finally, hypomethylation levels in non-tumorous tissues above the median value was nearly significantly associated with reduced recurrence-free survival. It is both biologically plausible and intuitive to recognize that epigenetic changes in precancerous lesions might be more useful clinical informants for identifying patients at-risk for developing earlier recurrences. Clinically detectable hepatocellular carcinomas represent a tissue type of extraordinary epigenetic and genetic aberrations. From the context of a myriad of molecular changes, tumors have progressed far beyond the non-tumorous counterpart from the surgical margin—tissue that appears histopathologically normal. Thus, epigenetic changes in non-tumorous tissue are inherently earlier events preceding a clinically detectable hepatocellular carcinoma, and it is perhaps not surprising epigenetic changes in the field—which will ultimately serve as the background from which additional tumors develop—are more associated with recurrence-free survival. We posit, then, that a careful investigation of the epigenetic and gene expression changes in the liver remnant can enhance the molecular information from tumorous tissues and other, more conventional clinicopathological features.

Our study has combined epigenetic analyses with whole genome microarray data. Because it is well-recognized that promoter hypermethylation may result in repression of the associated transcript, we aimed to characterize the relationship between methylation of specific genes and corresponding mRNA levels. We hypothesized that tissues in which gene promoters were methylated would also show decreased expression. Even though we found

significant differences in gene expression between tumorous and non-tumorous tissues for all of the genes but *APC*, the univariate Cox proportional hazard analyses showed that gene expression of these 7 genes was not associated with recurrence-free survival, with the exception of *SOCS-1* expression in non-tumorous samples.

RIZ1 and *GSTP1* were the only genes whose expression was significantly lower in methylated samples when examining both tumorous and non-tumorous samples together. Counter-intuitively, expression of $P16^{INK4A}$ was actually significantly higher in samples showing $P16^{INK4A}$ methylation. When analyzed separately in the tumorous cohort, this relationship was upheld. Additionally, *MGMT* expression was significantly lower in methylated samples in the non-tumorous cohort. Though *RIZ1* methylation was significantly associated with reduced recurrence-free survival and others have found a correlation between *RIZ1* hypermethylation and reduced mRNA levels in tumorous tissues (47), our study did not confirm this. The lack of the expected correlation between promoter hypermethylation and gene expression in 5 of the 7 genes analyzed could be the result of examining only one CpG island per gene promoter, and not comprehensively analyzing methylation in the entire promoter region. Furthermore, promoter hypermethylation is just one biological modification affecting gene expression. Because mRNA levels are quite dynamic, it is reasonable to hypothesize that other compensatory processes are operating as cells attempt to reverse changes in expression due to promoter hypermethylation. In the dysregulated biological and molecular milieu of cancer, a host of mechanisms could be responsible for achieving this, including miRNA binding, chromatin and histone modifications, and larger chromosomal irregularities, such as loss of heterozygosity.

To further investigate the apparent lack of a relationship between methylation status and gene expression, we adopted the converse approach, and selected genes for methylation analysis based on expression profiles of genes which were related to recurrence-free survival. Except for *GATA-4,* all of the nine genes identified with putative promoter CpG islands had not previously been reported in the literature, and thus were novel targets for analysis. However, none of these genes were methylated in 8 tumorous or 8 non-tumorous tissues following MSP analyses, including *GATA-4,* which was methylated in 58% of HCC cases in reports by others (25). Thus, overall, we conclude that there is lack of agreement between promoter methylation status and gene expression in a cohort of HCV-positive Japanese HCC patients, and we reason that assessment of methylation status alone is incapable of explaining the downstream cascade of events leading to dysregulated gene expression in HCV-associated hepatocarcinogenesis.

However, we must caution that the weak relationship demonstrated between promoter methylation status and downstream gene expression changes might also be accounted for by the tissue procurement procedure. The samples used in this study were not micro-dissected. Rather, they were whole tissue samples and thus represent a heterogeneous tissue with respect to the cellular sub-populations of cells (e.g., epithelium and the stroma). Consequently, overall observed gene expression changes can be regarded as the net contribution of gene expression changes in both the epithelial cells of the tumor and the supportive non-epithelial cells. It is conceivable that up-regulation of gene expression in stromal cells could have offset some of the decreases in gene expression due to promoter hypermethylation, as this could have occurred only in epithelial cells of the cancer.

From a genome-wide perspective, then, it appears that gene expression signatures of recurrence-free survival are more difficult to characterize, because multiple other mechanisms that influence gene expression are likely operating, perhaps undetectably, and potentially in different cellular sub-populations. By focusing within a smaller, more gene-specific context, however, patterns of methylation appear to be more stable and robust markers of the sequential accumulation of molecular aberrations along multistep hepatocarcinogenesis. Biomarkers such as *RIZ1* hypermethylation in non-tumorous tissues may serve a more clinically useful role, as its association with earlier recurrence or as an early event in tumorigenesis is reproducible between studies (45; 47).

In summary, we examined some of the epigenetic changes incurred during HCV-related hepatocarcinogenesis and demonstrate the technical and practical challenges of relating promoter methylation status to corresponding gene expression levels and recurrence-free survival. Multiple genes in multiple pathways known to be improperly regulated during tumorigenesis were hypermethylated in both tumorous and non-tumorous tissues. Additionally, global changes in DNA hypomethylation were more pronounced in tumorous tissues. Together, our epigenetic data establishes an aberrant methylation profile in a cohort consisting of an etiologically distinct sub-group of HCV-positive HCC cases. The finding that *RIZ1* methylation and increased levels of LINE-1 hypomethylation in non-tumorous tissues are associated with recurrence-free survival underscores the importance of assessing the epigenetic state of the liver remnant following tumor resection.

Study Limitations

Several features of this research project potentially limited the scope, applicability, and definitiveness of the aforementioned results. Perhaps the biggest limitation was the small sample size, which included only 43 tumorous tissues, 45 non-tumorous tissues, and 39 pairs from the same patient. Though the vast majority of literature on aberrant methylation in HCC uses patient cohorts that are less than 100 patients in size, one must acknowledge the low statistical power afforded by such a small sample size. In particular, dichotomizing cases by methylation status for most of the genes, both within the tumorous and non-tumorous sub-cohorts, resulted in fewer than 10 cases in a group. The same limitation applies for the analyses of recurrence-free survival using Kaplan-Meier curves or Cox proportional hazards modeling. A larger sample size could have potentially increased the survival separation between methylated and unmethylated samples, uncovering more statistically significant relationships. However, procuring fresh-frozen tissue samples from HCC resection patients is always a difficult endeavor.

The small number of genes analyzed for methylation status (seven) also limited the scope of this study. Because there were clearly differences in the frequency of methylation according to different genes (e.g., 0% methylation of *MGMT* versus 100% methylation of *RASSF1A* in tumorous tissues), adding several more genes might have revealed a pattern of differential methylation according to the gene being analyzed (e.g., a group of genes with low, moderate, or high methylation frequencies in tumorous or non-tumorous tissues).

Methodologically, the MSP used in the analyses of gene-specific promoter methylation was inherently qualitative, in that it yielded dichotomous data (methylated or not methylated). Real-time, quantitative MSP has only recently become a reliable method for

investigating methylation in cancer, and thus simple MSP is still frequently used in epigenetic studies of HCC. However, the use of quantitative MSP could expose differences in methylation according to tissue type, recurrence-free survival, or other clinicopathological variables that are perhaps indiscernible with simple MSP.

Further Avenues of Research

Future directions of research generated from the present project would include the addition of at least 20 more genes for MSP analysis. To investigate the relationship between HCV-associated ROS production and HCC (9), several of these genes would be related to mechanisms of ROS scavenging and/or DNA adduct repair. A quantitative, real-time MSP assay would replace the simple qualitative one. This technique would generate far more quantitative data, however, and one would probably need to enlist the services of expert bioinformaticists/biostatisticians. Recognizing that normal aging livers can exhibit detectable levels of DNA methylation through this technique (32), this improvement would require age-matched control DNA to allow meaningful comparisons between levels of methylation and to ensure conclusions regarding the methylation status was due to pathology and not simply an artifact of natural aging. Hierarchical clustering of the level of gene-specific methylation could potentially produce easily identifiable patterns in methylation levels according to the tissue type or other pre-selected variables, and pathway analyses of the implicated genes could be performed.

The addition of tissues obtained from HCV-positive, HBV-negative cirrhotic livers would allow the assessment of the sequential epigenetic changes as the liver pathology progresses from normal (but aging) to cirrhotic, to the non-neoplastic margin, to HCC, and

would permit more explicit characterization of the epigenetic alterations accompanying HCV-associated HCC.

To bolster data obtained from MSP analyses, this project could expand to include immunohistochemistry experiments to test for the protein products of the cancer-related genes. If the functional consequence of promoter hypermethylation is reduced gene expression, and hence, reduced protein product, then the demonstration of correspondingly less protein in methylated tissues could validate the results of the methylation status.

Lastly, because promoter hypermethylation is just one epigenetic mechanism involved in hepatocarcinogenesis, this project would be enhanced by an investigation into other epigenetic changes that might be associated with recurrence-free survival, such as determining histone acetylation status using chromatin immunoprecipitation assays.

Concluding Remarks

That carcinogenesis is a complex, multistep process of both epigenetic and genetic aberrations has been recognized for decades. This study provides results that seem to add an additional layer of intricacy to the molecular enigma of hepatocarcinogenesis. It is manifest both from the high frequencies of gene methylation in non-tumorous tissues and from the numerous relationships found between epigenetic changes in non-tumorous tissues, clinicopathological variables, and recurrence-free survival that preoplastic tissue from the surrounding margin is severely compromised in HCV-positive HCC patients. Because HCC tumor recurrence in patients undergoing curative resection surgery is particularly rapid and unavoidable, the field of hepatocarcinogenesis research must continue to focus on identifying and characterizing biomarkers of liver pathology that offer *prospective* utility. The capacity to detect aberrant promoter methylation in serum DNA several years before clinical detection

of HCC, and the power of this methylation status for predicting HCC (37) inspires hope in

the quest for earlier HCC detection, earlier treatment, and longer survival in patients afflicted

by this devastating cancer.

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