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Gene Expression Variations in Microsatellite Stable and Unstable Colon Cancer Cells

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Abstract

Background—Microsatellite instability (MSI) is a marker of chemoresistance, but it is associated with improved survival when compared to microsatellite-stable (MSS) colon cancers. We hypothesized that MSI tumors over-express chemoresistance-associated genes and under-express DNA damage/repair genes. We used ultra high-throughput sequencing (UHTS) to assess the expression of representative genes in MSI and MSS colon cancer cell lines.

Methods—Solexa UHTS was used to examine gene expression in HCT116 (MSI) and HT29 (MSS) cells, and normal colonic mucosa (NCM). We compared expression of 40 genes involved in chemoresistance, DNA repair, DNA damage, and drug metabolism pathways.

Results—We observed gene expression differences between MSI and MSS cell lines in 8 out of 40 genes involved in mismatch repair (MMR), DNA repair, drug metabolism and chemoresistance. MMR gene expression was lower in MSI cells, which is consistent with the MSI phenotype, whereas DNA repair genes were highly expressed in these cells. Genes associated with chemoresistance and drug metabolism also had increased expression in MSI cells. No difference in expression of DNA damage genes was observed between MSI and MSS cell lines.

Conclusion—Using UHTS gene expression analysis, we identified differential expression of genes between MSI and MSS cell lines which may account for resistance to chemotherapy in MSI tumors. UHTS expression analysis has the potential to identify genome-wide predictors of response or resistance to chemotherapy.

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Keywords

Microsatellite stable; microsatellite unstable; colon cancer; Solexa; gene expression; ultra-high throughput sequencing; DNA repair genes; DNA damage genes; 5-fluorouracil

Introduction

Approximately 15% of colorectal cancers are characterized with microsatellite instability (MSI) (1-11). These MSI colorectal tumors represent a distinct pathway of pathogenesis and genomic instability and are generally resistant to 5-fluorouracil (5-FU) based chemotherapy (6, 8-10, 12-15). This resistance has been attributed to deficient DNA mismatch repair (MMR) (8-10, 16). MSI tumors are typically located in the ascending colon with poor histological differentiation, mucin production, and lymphocytic infiltration (1, 6, 9, 12, 13). Although the clinical features of MSI tumors may be associated with a poor prognosis, these tumors are often associated with a better prognosis compared to microsatellite stable (MSS) tumors (2, 3, 6, 8, 9, 17). It is not known how deficiencies in the MMR pathway, which is a hallmark of MSI tumors, contribute to chemoresistance. Previous studies have focused on global patterns of gene expression rather than specific pathways that may contribute to treatment resistance, such as DNA repair pathways, DNA damage signaling, 5-FU drug metabolism, and chemoresistance genes (4, 5, 7).

Ultra high-throughput sequencing (UHTS) technology provides comprehensive transcriptome analysis, which can identify differences in gene expression by quantifying the exact number of gene transcripts present (18-27). Other methodologies, such as microarray assays only semi-quantitatively detect differences in gene expression levels (25, 26). Therefore, UHTS can accurately quantify levels of expression and characterize the expression of the entire genome, including genes of low abundance that cannot be quantified by microarray analysis.

Our objective was to identify differential gene expression in signaling pathways involved in DNA repair, DNA damage, 5-FU metabolism, and chemoresistance to identify potential genetic differences in chemotherapy-associated pathways between MSI and MSS colon cancer cells. We used the innovative Illumina Solexa expression assay which utilizes UHTS technology to compare RNA transcripts between MSI and MSS colon cancer cell lines to quantify gene expression levels and determine whether differences exist between MSI and MSS cells.

Methods

Cell lines and cultures

HCT116 and HT29 colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), and normal colonic mucosa (NCM) cells were obtained from biopsy samples from normal colonic mucosa. All cell lines were grown in RPMI 1640 media and supplemented with 10% fetal bovine serum (FBS). Cell cultures were maintained at 37°C in an incubator with 5% CO₂.

Colon cancer cells and exposure to 5-FU

Colon cancer cells (HCT116 and HT29) were seeded (3×10^3) in a 96-well plate. Cells were allowed to adhere overnight and then treated with 5-FU (1, 3, 5, and 10 μ M). Experimental and untreated control cells were both maintained in growth medium supplemented with 10% FBS. Following exposure to 5-FU (24 and 48 h), colon cancer cells were lifted, counted, and

assessed by the trypan blue exclusion method for cell viability. These assays were performed in triplicate.

DNA extraction and establishment of microsatellite status

Genomic DNA was extracted from colon cancer cell lines using the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Chatsworth, CA). The microsatellite status of the cells was determined using five mononucleotide markers; BAT25, BAT26, NR21, NR24 and MONO27 (MSI Analysis System, Promega; Madison, WI). Each PCR reaction contained 7.85 μ L nuclease free water, 1 μ L GoldSTAR 10 $\times$ Buffer, 1 μ L 10 $\times$ multiplex primer mix, and 0.15 μ L AmpliTaq Gold DNA Polymerase. Lastly, 2 μ L of sample DNA was added for a total reaction volume of 12 μ L.

Genotyping was performed with the ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) and data analysis was performed using GeneMapper software (Applied Biosystems). Tumors were classified as either microsatellite unstable (MSI) or microsatellite stable (MSS) as per standard definitions outlined by Boland et al. (1).

RNA isolation and Solexa sequencing

Total RNA was isolated from HCT116 and HT29 cell lines, and NCM cells using the QIAgen RNeasy Mini Kit (Qiagen). RNA extracts were submitted for transcriptome analysis by Solexa sequencing (Illumina Inc., San Diego, CA). Genome-wide transcriptome analysis was performed by UHTS to quantitatively measure gene expression levels. Solexa sequencing was performed on HCT116 and HT29 cells, and NCM cells. A transcriptome library was compiled for all genes expressed in each cell line. We further assessed genes associated with DNA repair, DNA damage, drug metabolism, and chemoresistance.

Statistical analysis

Expression levels of genes associated with DNA repair, DNA damage, drug metabolism and chemoresistance in HCT116 and HT29 cells were compared to NCM cells. The fold-changes in gene expression in colon cancer cells compared to NCM cells were quantified as a $\log_2$ ratio (expression in cancer cells/expression in NCM cells). Subsequently, gene expression cut-offs were designated as high (> 2-fold increase) or low (< 0.5-fold increase) expression compared to NCM cells, as determined by prior publication (28). These cut-offs for evaluating gene expression have been shown to be appropriate for detecting statistically significant differences ($P < 0.05$) in gene expression.

Results

Confirmation of MSS or MSI genotype

The genotype of each cell line was first determined. HCT116 cells were confirmed to have the MSI genotype by utilizing the panel of five markers, and HT29 was determined to be MSS. Two populations of NCM cells were also assessed for MSI, and confirmed to have the MSS genotype.

Colon cancer cells and resistance to 5-FU

Cell viability for colon cancer cells (HCT116 and HT29) was assessed following exposure to 5-FU *in vitro*. After 24 h of exposure with 5-FU, we observed no difference in the number of viable cells between HCT116 and HT29 cell lines. After 48 h, there was significantly increased cell death in HT29 cells compared to HCT116 cells (70% versus 39%) (Figure 1). These results indicate that HCT116 colon cancer cells are more resistant to 5-FU treatment than HT29 colon cancer cells.

5-Fluorouracil Metabolism Genes

Clinically, MSI tumors do not respond to 5-FU based chemotherapy; as such, we examined genes involved in 5-FU metabolism in HCT116 and HT29 cells (Table 1). Of the genes involved in 5-FU metabolism, the uridine phosphorylase 1 (UPP1) gene showed increased expression in HCT116 cells and decreased expression in HT29 cells compared to NCM cells. In contrast, no differences in the expression levels of 5-FU metabolism genes, such as thymidylate synthetase (TYMS), dihydrofolate reductase (DHFR), dihydropyrimidine dehydrogenase (DPYD), thymidine phosphorylase (TYMP), and uridine monophosphate synthetase (UMPS) were observed between HCT116 and HT29 cells when compared to NCM cells.

Mismatch Repair Genes

MSI tumors are characterized by their deficiency in DNA mismatch repair. We examined the expression of mismatch repair (MMR) genes: MLH 1 and 3, MSH 2, 3 and 6, and PMS2 (Table 2). The expression of MLH1 in HCT116 cells was down-regulated compared to HT29 cells, which is consistent with previous studies (29). The relative expression of the MSH3 gene was also decreased in HCT116 cells but was elevated in HT29 cells. Expression levels of MSH2, MSH6, and PMS2 were similarly increased in both HCT116 and HT 29 cells.

DNA Repair Genes

By definition, MSI tumors lack the ability to repair mismatches in DNA base-pairing, and thus alternative DNA repair pathways instead may contribute to treatment resistance. DNA repair genes were further analyzed in our study (Table 3). X-ray repair cross-complementing protein 1 (XRCC1) is a gene involved in base excision repair (BER) and functions to repair DNA single stranded breaks introduced by anti-metabolite based chemotherapies, such as 5-FU (30-33). The expression levels of XRCC1 were highly elevated in both HCT116 and HT29 cells compared to NCM cells. Other genes associated with BER, such as DNA ligase 3 (LIG3), also showed increased expression in HCT116 and HT29 cell lines (Table 3).

Additional DNA repair pathways were also examined. Nucleotide excision repair (NER) is responsible for removing damaged or replaced nucleotides; (31-33) thus, genes associated with NER were examined (Table 3). Excision repair cross-complementing group (ERCC) 1, 4 and 6 showed increased expression in HCT116 cells compared to NCM cells. This contrasts levels observed in HT29 cells, which demonstrated similar expression levels to NCM cells. However, not all genes involved in NER showed differences in expression levels between HCT116 and HT29 cells. The pattern of expression levels of ERCC2, 3, 5, and xeroderma pigmentosum complementation group A and C (XPA and XPC) in HCT116 and HT29 cells were similar when each was compared to NCM cells.

Platinum-based chemotherapy, including agents such as oxaliplatin, and topoisomerase inhibitors, such as irinotecan, cause DNA double-stranded breaks that are repaired mainly by two pathways; homologous recombination (HR) and non-homologous end joining (NHEJ) (31-33). DNA repair genes involved in HR and NHEJ pathway, such as XRCC 2, 3, 4 and 6, were highly expressed in both HCT116 and HT29 cells compared to NCM cells (Table 3). DNA repair protein RAD51 (RAD51) and serine-protein kinase ATM (ATM) expression levels in HCT116 and HT29 were also elevated compared to NCM cells. Breast cancer 1 gene (BRCA1) which also participates in DNA damage signaling (34) showed increased expression levels in both HCT116 and HT29 cells.

Genes Associated with DNA Damage

The function of most chemotherapeutic regimens is to cause DNA damage which leads to cell death (32, 33). Seven genes involved in signaling DNA damage response showed elevated expression in both HCT116 and HT29 cells in comparison to NCM cells (Table 4). Serine/threonine-protein kinase ATR (ATR) is associated with a DNA damage response pathway that involves genes such as checkpoint homolog 1 (CHEK1) and retinoblastoma binding protein 8 (RBBP8). Two genes which encode proteins that interact with p53, tumor suppressor p53-binding protein 3 (TOPORS) and cell division cycle 25 homolog C (CDC25C), were also highly expressed in HCT116 and HT29 cells. In addition, expression of RAP1 interacting factor homolog 1 (RIF1) and structural maintenance of chromosomes 1A (SMC1A) genes, which encode proteins that interact with DNA, were increased in both cell lines compared to NCM cells. Although expression of genes responsible for DNA damage signaling was up-regulated in comparison to normal cells, no difference in expression was observed between MSI and MSS cell lines.

Chemoresistance Genes

Differences in gene expression of chemoresistance-associated genes may explain the clinical behavior observed in MSI cells. HCT116 cells showed differential expression of two genes involved in resistance to chemotherapy (Table 5). ATP-binding cassette subfamily C2 (ABCC2) and hypoxia inducible factor 1 alpha (HIF-1A) showed greater than 2-fold increased expression in HCT116 cells compared to NCM cells. HT29 cells showed similar expression levels of ABCC2 and HIF-1A genes as NCM cells. Expression levels of ATP-binding cassette subfamily B1 (ABCB1) and carboxylesterase 1 (CES1) were decreased in both HCT116 and HT29 cells compared to NCM cells. These genes have also been implicated in resistance to chemotherapy (35-38).

Discussion

Utilizing UHTS, we demonstrated that several pathway-specific genes are differentially expressed between two established colon cancer cell lines. Our observations show that differential expression of genes involved in DNA repair pathways and chemoresistance genes are consistent with the known clinical phenotype of MSI tumors (7, 10, 16, 33, 37, 39, 40). As such, UHTS is a powerful technique which can characterize tumors by their unique gene expression profile. Such comprehensive data can be utilized to further characterize candidate genes of treatment response or chemoresistance.

UHTS technology overcomes the limitations of microarrays, and genome-wide analyses of gene expression in tumor cells can now be performed to examine precise changes in levels of expression. UHTS directly quantifies the abundance of gene transcripts through next generation sequencing. This is advantageous as it avoids the high background noise resulting from non-specific probe hybridization present in microarray analysis, and allows for the sensitive detection of genes with low expression. Further uses of UHTS technology have been employed to identify unique transcription factor binding sites, detect areas of DNA methylation, and analyze the expression of small RNAs (20, 22, 24-26, 35). In our study, we utilized a novel technology to perform a focused examination of pathways and genes that may contribute to chemoresistance, in order to characterize the clinical phenotype observed in MSI tumors.

Two possible mechanisms can contribute to resistance to chemotherapy; direct or indirect. Direct pathways include mechanisms that process the chemotherapeutic agent. These typically involve drug metabolism pathways and mechanisms that influence drug delivery. Indirect pathways involve processes that respond to the effects of chemotherapeutic agents,

such as DNA repair and DNA damage pathways. Both mechanisms may contribute to how MSI tumors fail to respond to 5-FU based chemotherapy. Here, we demonstrated a differential expression pattern for genes involved in 5-FU metabolism and chemoresistance between MSI and MSS tumors, including UPP1. Our results are inconsistent with prior studies which suggest a role for UPP1 in promoting the cytotoxic effects of 5-FU (41-44). Cao et al. demonstrated that the induction of UPP1 enhances the cytotoxic effects of the 5-FU pro-drug 5'-deoxy-5-fluorouridine (41). Therefore, the resistance to 5-FU observed with MSI tumors may in fact be unrelated to UPP1. We also observed that MSI tumors have increased expression of two genes, HIF-1A and ABCC2, which may influence the delivery of 5-FU to MSI tumors. Up-regulation of HIF-1A induces expression of drug efflux transporters, alters activity of DNA repair pathways, and promotes cell survival, which jointly counter the effects of chemotherapy (40). Recent studies also demonstrated the role of ABC transporters in multi-drug resistance (38). Currently, no clinical trials analyzing tumors with the MSI genotype have determined whether these tumors have increased levels of HIF-1A or ABCC2. However, we suspect that the expression of these genes may underlie, at least in part, the observed chemoresistance of MSI tumors.

MSI tumors have deficient MMR pathways. It is not yet clear as to how decreased MMR function results in 5-FU resistance. We demonstrated differential expression of alternate DNA pathways, particularly the NER pathway. The NER DNA repair pathway may play a larger role than other DNA repair pathways such as NHEJ and HR in reversing the damage caused by 5-FU. We showed that HCT116 cells have increased expression of genes involved in NER when compared to NCM cells. DNA damage signaling can mediate repair pathways, inhibit cell cycle progression, and signal apoptosis. No distinction between MSI and MSS cell lines could be found from the differential expression of these DNA damage signaling genes, suggesting that these genes may not contribute to the chemoresistance observed in MSI tumors.

Microsatellite instability is one of the earliest described molecular alterations in colorectal cancer (3, 12, 13). We showed that HCT116 cells, which harbor the MSI genotype, have differential expression of genes associated with DNA repair pathways, chemoresistance genes, and 5-FU metabolism. These studies demonstrate the utility of UHTS for detecting pharmacogenetic differences in cancer cells. This technology provides a powerful approach to better understand genes involved in chemotherapeutic response and resistance and could be used to investigate other MSI and MSS cell lines to identify additional genes and signaling pathways related to 5-FU-based therapies. Ultimately, this technology may lead to optimized and individualized therapy for patients with rectal cancer.

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Figure 1. Cell viability after treatment with 5-FU for 48 hours

Viable cells present in HCT116 and HT29 cells after treatment with 5-FU for 48 hours.

Table 1
Expression Levels of Genes Involved in Drug Metabolism of 5-Fluorouracil
Chemotherapy

Gene	HCT 116	HT 29
DPYD	↓	↓
TYMP	↓	↓
TYMS	↑	↑
DHFR	↑	↑
UMPS	↑	↑
UPP1	↑	↓

↑ = Expression level > 2× normal colonic mucosal cells; ↓ = Expression level < ½ normal colonic mucosal cells.

Table 2
Expression Levels of DNA Mismatch Repair Genes

Genes	HCT 116	HT 29
MLH1	↔	↑
MLH3	↔	↔
MSH2	↑	↑
MSH3	↓	↑
MSH6	↑	↑
PMS2	↑	↑

↑ = Expression level > 2× normal colonic mucosal cells; ↓ = Expression level < ½ normal colonic mucosal cells; ↔ = Expression level equal to normal colonic mucosal cells.

Table 3
Expression Levels of DNA Repair Genes

Repair Pathway	Gene	HCT 116	HT 29
BER	XRCC1	↑	↑
	LIG3	↑	↑
NER	ERCC1	↑	↔
	ERCC2	↑	↑
	ERCC3	↑	↑
	ERCC4	↑	↔
	ERCC5	↔	↔
	ERCC6	↑	↔
	XPA	↑	↑
	XPC	↑	↑
HR / NHEJ	XRCC2	↑	↑
	XRCC3	↑	↑
	XRCC4	↑	↑
	XRCC6	↑	↑
	ATM	↑	↑
	RAD51	↑	↑
	BRCA1	↑	↑

Abbreviations: BER, Base excision repair; NER, Nucleotide excision repair; HR, Homologous recombination; NHEJ, Non-homologous end joining.

↑ = Expression level > 2× normal colonic mucosal cells; ↓ = Expression level < ½ normal colonic mucosal cells; ↔ = Expression level equal to normal colonic mucosal cells.

Table 4
Expression Levels of DNA Damage Signaling Genes

Gene	HCT 116	HT 29
RBBP8	↑	↑
CDC25C	↑	↑
RIF1	↑	↑
SMC1A	↑	↑
TOPORS	↑	↑
ATR	↑	↑
CHEK1	↑	↑

↑ = Expression level > 2× normal colonic mucosal cells; ↓ = Expression level < ½ normal colonic mucosal cells.

Table 5
Expression Levels of Chemoresistance Genes

Gene	HCT 116	HT 29
ABCB1	↓	↓
ABCC2	↑	↔
CES2	↓	↓
HIF1A	↑	↔

↑ = Expression level > 2× normal colonic mucosal cells; ↓ = Expression level < ½ normal colonic mucosal cells; ↔ = Expression level equal to normal colonic mucosal cells.