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Effect of genomic instability and mutations on the signaling pathways in colon cancer cells

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1. ABSTRACT

Microsatellite instability (MSI) is present in about 15% of colorectal cancers and plays critical roles in the development and progression of these cancers. The goal of this study is to determine the global effect of microsatellite instability on the signaling pathways and network in colon cancer cells. We profiled the expression and phosphorylation of 110 proteins in six colon cancer cell lines by using Protein Pathway Array. The pathways and network constituted by these proteins were identified by using Ingenuity Pathway Analysis. Our results showed that 25 proteins and phosphoproteins change more than 1.5-fold between MSI and microsatellite stable (MSS) cells. Sixteen major pathways were affected in MSI cells, including p53 and 14-3-3β pathways, with p53 and HGF being the most important pathways. Finally, although the EGFR/K-RAS/MEK pathway was not affected in MSI cells, collateral pathways such as the p70S6K and p90RSK pathways were activated in MSI cells. Thus, suppression of the p53 pathway and activation of the HGF pathway in MSI cells may be critical in the tumorigenesis of MSI colorectal cancer.

2. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer diagnosed in the United States and the second leading cause of cancer death (1). Two different genetic pathways contribute to the development and progression of colorectal cancer. The most frequently involved pathway is the chromosomal instability pathway (CIN), which is also called microsatellite stable (MSS). The MSS phenotype is associated with loss or gain of chromosome arms, chromosomal translocations, or gene amplification, and is characterized by alterations in tumor suppressor genes and oncogenes, including *APC*, *TP53*, and *K-RAS* (2). On the other hand, 10%–15% of CRC show microsatellite instability (MSI), which is strongly associated with mutations in specific oncogenes and tumor suppressor genes, especially *BRAF* and *MRE11A*, and others such as *K-RAS*. Several clinical studies showed that MSI colon cancer has a more favorable prognosis and is less prone to lymph node and distance metastasis (3). Furthermore, the MSI phenotype may predict the response to treatment with 5-fluorouracil (5-FU) and irinotecan (2).

MSI results from a germline mutation in the mismatch repair (MMR) system or somatic hypermethylation of the promoter region of the *MLH1* or *MSH2* gene (2, 4). The MMR system is responsible for the surveillance and correction of errors introduced in microsatellites. Tumors with MMR deficiency exhibit frequent errors in microsatellite DNA, which are short segments of DNA containing tandem repeats of mono-, di-, or trinucleotides (2), leading to frameshift mutations in tumor suppressor genes or oncogenes. More than 30 genes have mutations that arise in microsatellite repeats in MMR-deficient tumors, and these genes are implicated in diverse cellular functions and pathways. For example, high frequencies of mutations and deletions were found in *PIK3CA, TP53, TGF-β, IGF, Bax* and *HDAC2* genes (2). Recent gene expression studies also revealed alteration of the apoptotic and immune response pathways in MSI cells (2, 5). However, the role of these pathways in the carcinogenesis of CRC and the interaction of these pathways in MSI CRC cells remain to be determined.

To further explore changes in the levels of protein and phosphorylation as well as alteration of pathways in MSI cells, we profiled 112 proteins/phosphoproteins in two colon cancer cell lines with MSI by Protein Pathway Array, and compared them with those in the four colon cancer cell lines with MSS. Protein Pathway Array is a high-throughput proteomic technique that combines multiplex immunoblot with computational analysis and allows measurement of the proteins and protein phosphorylation involved in signaling pathways, cell cycle progression, survival, and apoptosis (6). Our results showed that 16 major pathways were affected in MSI cells, including p53, PI3K/AKT, 14-3-3β, ATM, and HGF, and further confirmed diverse alteration of pathways in MSI CRC cells.

3. MATERIALS AND METHODS

3.1. Cell culture

Human colon adenocarcinoma cell lines, namely, HCT15, HCT116, HT29, SW620, SW480, and Caco-2, were purchased from the American Type Culture Collection (Rockville, MD). HCT116 and HT29 cells were cultured routinely in McCoy's 5a modified medium supplemented with 10% fatal bovine serun (FBS), 100 units/mL penicillin, and 100 g/mL streptomycin. SW480 and SW620 cells were cultured routinely in Leibovitz's L-15 Medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 g/mL streptomycin. Caco-2 cells were cultured routinely in Eagle's minimum essential medium supplemented with 20% FBS, 100 units/mL penicillin, and 100 g/mL streptomycin. HCT15 cells were cultured routinely in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2, except for SW480 and SW620. SW480 and SW620 cells were cultured in 100% air without CO_2 supplementation.

3.2. Protein Pathway Array analysis

Total proteins were extracted from HCT15, HCT116, HT29, SW620, SW480, and Caco-2 cells by using 1× cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing 20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1μg/mL leupeptin in the presence of 1× proteinase inhibitor and 1× phosphatase inhibitor cocktails (Roche Applied Science, Indianapolis, IN). The lysate was sonicated three times for 15 s each time, and then centrifuged at 14,000 rpm for 30 min at 4°C. The protein concentration was determined with the BCA Protein Assay kit (PIERCE, Rockford, IL). Lysate (300 μg) was loaded in one well across the entire width of a 10% SDS polyacrylamide gel and separated by electrophoresis as described previously (7, 8). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), which was then blocked for 1 h with blocking buffer that included either 5% milk or 3% BSA in 1×TBST containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20. Next, the membrane was clamped on a western blotting manifold (Mini-PROTEAN II Multiscreen apparatus; Bio-Rad) that isolates 20 channels across the membrane. Multiplex immunoblotting was performed using a total of 110 protein-specific or phosphorylation site-specific antibodies. Three sets of antibodies (a total of 36–38 protein-specific or phosphorylation site-specific antibodies per set) were individually used for each membrane, and all of the antibodies (from various companies) were validated independently before inclusion in Protein Pathway Array. For the first set of 36 primary antibodies, a mixture of two antibodies in blocking buffer was added to each channel, and then incubated at 4°C overnight. The membrane was then washed with 1×TBS and 1×TBST, and further incubated with secondary anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase (Bio-Rad) for 1 h at room temperature. The membrane was developed with chemiluminescence substrate (Immun-Star™ HRP Peroxide Buffer/Immun-Star™ HRP Luminol Enhancer; Bio-Rad), and chemiluminescent signals were captured using the ChemiDoc XRS System (Bio-Rad). The same membrane was then stripped using stripping buffer (Restore™ Western Blot Stripping Buffer; Thermo Scientific, Rockford, IL), and then used for detection with a second set of primary antibodies as described above.

For Protein Pathway Array data analysis, the correct band of each protein was identified visually, and the signal of each protein was determined by densitometric scanning (Quantity One software package, Bio-Rad). The background was locally subtracted from the raw protein signal, and the background-subtracted intensity was normalized by the "global median subtraction" normalization method to reduce variations arising from different runs (such as transferring and blotting efficiency, total protein loading amount, and exposure density). In detail, for each protein, its intensity was divided by the total intensities of all proteins in the same blot membrane, and then multiplied by the average intensities of each protein in all samples. The normalized data were used in the subsequent statistical analysis. Three independent assays were performed for each cell. Only those proteins that showed consistent and substantial (average > ±1.5-fold)

Table 1. Genetic characteristics of 6 colon cancer cell lines

Cell Line	MSI status	K-ras	B-raf
HCT15	MSI	Mut	Wt
HCT116	MSI	Mut	Wt
HT29	MSS	Wt	Mut
SW620	MSS	Mut	Wt
SW480	MSS	Mut	Wt
Caco-2	MSS	Mut	Wt

changes between conditions were selected for subsequent analysis.

3.3. Antibodies used in this study

The following antibodies, obtained from Cell Signaling Technology, Inc. (Beverly, MA), were used for immunoblotting: polyclonal antibodies to p-EGFR (Tyr1148), p-HER2/ErbB2 (Tyr1221/1222), p-PDK1 (Ser241), p-PKCα/bII (Thr638/641), p-PKCδ(Thr505), p-p53 (Ser392), p-AKT (Ser473), p-PTEN (Ser380), p-Rb (Ser780), p-β-catenin (Ser33/37/Thr41), p-Stat5 (Tyr694), p-Stat3 (Ser727), p-ERK (Thr202/Tyr204), p-c-Kit (Tyr719), p-eIF4B (Ser422), p-ERK5 (Thr218/Tyr220), p-p90RSK (Ser380), p-CREB (Ser133), p-mTor (Ser2448), Phospho-IGF-I Receptor β (Tyr1131)/Insulin Receptor β (Tyr1146), p-cdc2 (Tyr15), p-c-Jun (Ser73), p-SAPK/JNK (Thr183/Tyr185), p-FLT3 (Tyr591), p-p38 (Thr180/Tyr182), ERK, Akt, c-kit, eIF4B, cleaved Caspase-3, VEGFR2, and p-GSK-3α/β (Ser21/9); and monoclonal antibodies to p-p70 S6 Kinase (Thr389), p-VEGFR Receptor2 (Tyr951), p-IKB (Ser32), p-Smad (Ser463/465), Notch4, CREB, Stat1, and PDGF Receptor-β. Polyclonal antibodies to p-Survivin (Thr 34), CyclinB1, CyclinD1, CyclinE, Cdk6, Cdc25B, Cdk2, BRCA1, Cdk4, Neu, 14-3-3β, PKCα, EGFR, Wee1, Cdc25C, Notch1, Trap, Bcl-2, HIF-1a, TTF-1, PTEN, SRC-1, p300, Bax, N-cadherin, Cdc42, TNF-α, Vimentin, Osteopontin, Survivin, E-cadherin, p16, WT1, NF-κB p52, NF-κB p50, Calretinin, p14 ARF, H-Ras, Bcl-6, NF-κB p65, and Myf-6 and monoclonal antibodies to β-tubulin, GAPDH, CHK1, MDM2, cdc2p34, E2F-1, PCNA, c-myc, FGFR-3, ETS1, p53, raf-1, p27, p21, p63, Mesothelin, ATF-1, K-RAS, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to Hsp90 was from Stressgen (Ann Arbor, MN). Polyclonal antibodies to TGF-β p-HGF R/c-MET (Y1234/1235), and p-HGF R/c-MET (Y1003) were from R&D Systems (Minneapolis, MN). Polyclonal antibodies to p-PKCα (Ser 657), HIF-3α, and HIF-2α were from Upstate (Billerica, MA), Abcam (Cambridge, MA), and Novus Biologicals (Littleton, CO), respectively. Monoclonal antibodies to XIAP and Cox2 were from BD Biosciences (Lexington, KY) and Cayman Chemical (Ann Arbor, MN), respectively. β-Actin, GAPDH, and β-tubulin were used as internal standards.

3.4. Signaling network analysis

Cell signaling networks were created using Ingenuity Pathway Analysis (IPA, Version 9.0) (http://www.ingenuity.com). Differentially expressed proteins identified by Protein Pathway Array were imported to IPA and mapped to their corresponding gene objects in the Ingenuity Pathways Knowledge Base. Proteins were categorized on the basis of their molecular functions by using the software, mapped onto genetic networks in the IPA database, and then ranked by score. The score associated with a particular network is the likelihood (i.e., negative log of a p-value) of the proteins identified as differentially expressed in a network being found together because of chance. The score is thus indicative of the proportion of proteins identified as differentially expressed in our analysis among all the molecules belonging to a particular network. In canonical pathways analyses, the canonical pathways that were most significant to the dataset were identified. The value assigned to the biological functions was presented in -log (p-value) by the IPA tool.

4. RESULTS

4.1. Genetic characteristics of colon cancer cell lines

Six human colon cancer-derived cell lines were used in this study. The genetic characteristics of these cells, including microsatellite instability, *EGFR* mutation, *K-RAS* mutation, and *B-RAF* mutation, are summarized in Table 1. For the *EGFR* gene, the two most common mutations, exon 19 deletion and L858R point mutation, were analyzed. For the *K-RAS* gene, codon 12 and 13 mutations were assessed. For the *B-RAF* gene, the V600E mutation was determined. The information on microsatellite stability was obtained from published literature (9, 10). The mutations in *EGFR*, *K-RAS*, and *B-RAF* genes were determined in our laboratory by using methods routinely used clinical diagnosis (PCR and sequencing method). HCT15 and HCT116 were microsatellite instable (MSI). In contrast, HT29, SW620, SW480, and Caco-2 were microsatellite stable (MSS). HT29 was the only cell line with a wild type *K-RAS* gene and a mutation in the B-RAF gene (V600E). All other cell lines possessed a mutation in the *K-RAS* gene, but none in the *B-RAF* gene. No mutations were detected in the EGFR gene in any cell line.

4.2. Comparison of protein expression between MSI and MSS cells

Of 110 proteins/phosphoproteins screened (Figure 1), 49 were detected in HCT15 and HCT116 cells (Figure 2). A few proteins such as PCNA and XIAP had relatively high-level expression in all cell lines, while other proteins such as Bcl6 and p-AKT had relatively low-level expression in all cell lines, suggesting their roles in colon tumorigenesis. Of 49 proteins and phosphoproteins, 25 showed a >1.5-fold change between MSI and MSS cells (Table 2). Of these 25 proteins, 17 showed a >1.5-fold increase in their expression in MSI cells: p-Smad1/5 (Ser463/465), Survivin, p-HGF R/c-MET (Y1234/1235), p-PKCα (Ser657), E-cadherin, PTEN, TNF-α, p-PTEN (Ser380), p-HGF R/c-MET (Y1003), p-p90RSK (Ser380), NF-κB p65, Cyclin E, cdc2 p34, p-β-Catenin (Ser33/37/Thr41), Bcl-6, p-p70 S6 Kinase (Thr389), and p-PKC α/βII (Thr638/641). In contrast, 8 proteins/phosphoproteins, 14-3-3β, p-p53 (Ser392), Cdk2, p53, raf-1, Cdk6, PKCα, and Hsp90, showed a >1.5-fold reduction in their expression in MSS cells.

4.3. Comparison of protein expression between K-RAS mutant cells and K-RAS wild-type cells with MSS

Of 110 proteins/phosphoproteins screened in MSS cells, 64 were detected in both K-RAS mutant cells

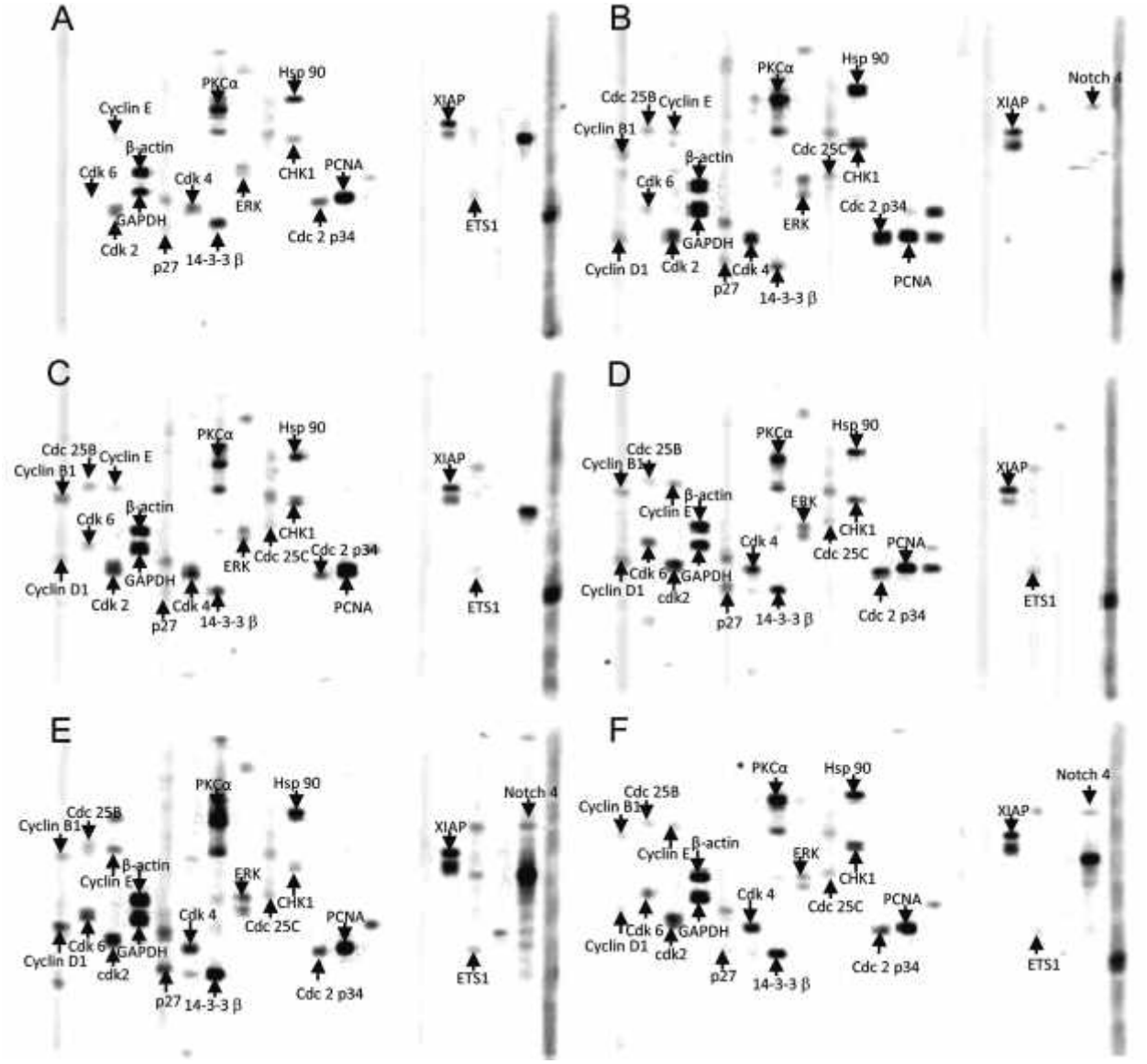

Figure 1. Representative autoradiographs showing expression and phosphorylation of the signaling-related proteins. The expression and phosphorylation levels of 110 proteins were determined by Protein Pathway Array. A: Caco-2; B: HCT15, C: HT29, D: HCT116, E: SW480, F: SW620.

(SW480, SW620, and Caco-2) and wild-type cells (HT29) (Figure 1). Of these, 35 proteins showed a >1.5-fold change between K-RAS mutant cells and wild-type cells (Table 3). Of these, 19 proteins, TNF-α, p-EGF Receptor (Tyr1068), ETS1, H-Ras, p-CREB (Ser133), p-PKCα (Ser657), β-catenin, p-p53 (Ser392), Hsp90, Akt, Cdk6, PKCα, Cyclin E, Survivin, 14-3-3β, Cdc2 p34, ERK, p27, and p-AKT (Ser473), showed a >1.5-fold increase in their expression in K-RAS mutant cells. In addition, 16 proteins/phosphoproteins, NFκB50, p14, Cdk2, E-cadherin, PTEN, Vimentin, p-HGF R/c-MET (Y1003), p-PKC α/βII, Cdc42, p-Stat3 (Ser727), p-eIF4B (Ser422), p-PKCσ (Thr505), p-p38 (Thr180/Tyr182), Notch4, ATF-1, and Calretinin, showed a >1.5-fold reduction in their expression in K-RAS mutant cells.

4.4. Canonical pathways altered in MSI cells and K-RAS mutant cells

To determine canonical pathways altered in MSI cells, we analyzed those proteins that were differentially expressed in MSI cells by using Ingenuity Pathway Analysis (IPA). Our results showed that 16 major pathways were significantly altered in MSI cells (Figure 3A). The top five pathways were the p53 pathway (-log (p-value) = 15.6), PI3K/AKT pathway (-log (p-value) = 12.9), 14-3-3β-mediated pathway (-log (p-value) = 11.6), ATM pathway (-log (p-value) = 9.48), and HGF pathway (-log (p-value) = 8.94). Interestingly, the EGFR signaling pathway was also affected in MSI cells with a -log (p-value) = 5.73 (ranked number 9). In contrast, 15 major pathways were significantly altered in K-RAS mutant MSS cells (SW480,

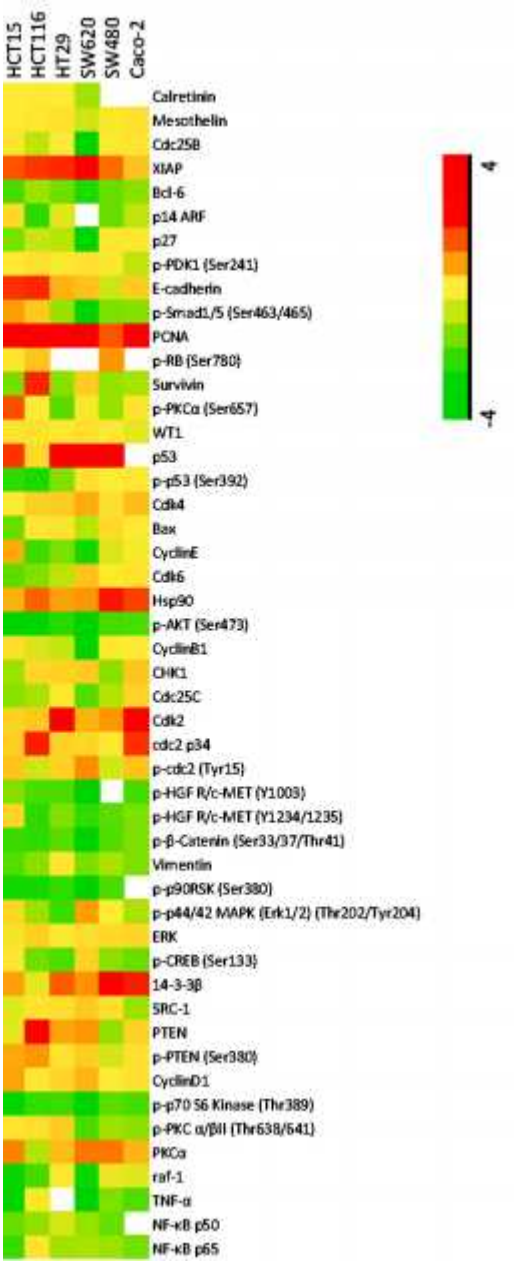

Figure 2. A heatmap showing the levels of proteins and phosphorylation in each cell. The value of expression or phosphorylation in each cell was transformed to Z-score, which ranged from 4 to -4. Green indicates decreased level and red indicates increased level.

SW620, and Caco-2) (Figure 3B). The top five pathways were the HGF pathway (-log (p-value) = 13.6), 14-3-3β-mediated pathway (-log (p-value) = 9.65), EGFR pathway (-log (p-value) = 9.48), PI3K/AKT pathway (-log (p-value) = 9.39), and p53 pathway (-log (p-value) = 8.47). As expected, the EGFR/K-RAS signaling pathway was significantly affected in K-RAS mutant cells (ranked number 3). To our surprise, the HGF and 14-3-3 pathways were also significantly affected.

4.5. The interactive network in MSI cells

To determine how these pathways and proteins interact with each other, we performed further network analysis using IPA. The proteins with a >1.5-fold change between MSI and MSS cells were included in IPA analysis. Our results showed a complex interactive network among these proteins and other putative proteins (Figure 4). Based on the number of connections among the proteins, several key nodes were identified, including p53, Cyclin D1, β-Catenin, and E-cadherin, with p53 being the most critical one in regulating the network. This complex network in MSI cells affects many cellular functions, including cell cycle progression, apoptosis, and survival.

4.6. Effect of MSI and K-RAS mutation on the EGFR pathway

Since the EGFR pathway was altered in both MSI and K-RAS mutant cells, we further investigated the changes of protein expression involved in the EGFR pathway. In MSI cells, Raf and 14-3-3β were down-regulated, while p70S6K and p90RSK were activated by phosphorylation (Figure 5A and Table 2). However, the down-stream effectors, i.e., ERK 1/2 and CREB, of the EGFR pathway did not change significantly. Interestingly, the PKCα pathway was activated by phosphorylation [p-PKCα (Ser657)], although total PKCα was reduced. These data suggest that the main EGFR/RAF/MEK pathway was not changed, but other collateral pathways such as p70S6K, p90RSK, and PKCα pathways were activated in MSI cells. In contrast, in K-RAS mutant MSS cells, Ras and ERK as well as 14-3-3β and p-CERB were up-regulated, while Raf, p70S6k, and p90RSk were not changed (Figure 5B and Table 3). In addition, PKCα was up-regulated via phosphorylation and at the protein level. These results suggest that alteration of the EGFR pathway was different in MSI and K-RAS mutant MSS cells: the EGFR/RAF/MEK pathway was activated in K-RAS mutant MSS cells, but not in MSI cells.

5. DISCUSSION

MSI accounts for the mutational activation and inactivation of cancer-related genes, those with positive and negative roles in cell growth or survival, which drive multi-step carcinogenesis. Our studies showed that 22.7% (25/110) of the proteins and phosphoproteins tested were significantly altered in colon cancer cells with MSI compared to colon cancer cells with MSS. These proteins are involved in 16 major pathways, including p53, PI3K/AKT, 14-3-3β, ATM, and HGF pathways (Figure 3). These proteins also interact to form a complex signaling network (Figure 4). Recent DNA sequencing analysis for mutation and deletion and microarray analysis for mRNA expression level confirmed alteration of some of these proteins and pathways (2). For example, the WNT signaling pathway is activated in MSI tumors through frameshift mutation of β-Catenin (11) because PKCα decreases (12). Frameshift mutation in the antiapoptotic gene Bax, which involves both p53 and 14-3-3β pathways, is observed in MSI tumors (13).

Table 2. Differential expression of proteins and phosphoproteins between MSI and MSS cells

Protein	Description	Ratio (MSI/MSS)
p-Smad1/5 (Ser463/465)	Transforming growth factor beta ligands to the nucleus	+7.1
Survivin	Inhibitor of apoptosis (IAP) family	+6.7
p-HGF R/c-MET (Y1234/1235)	Essential for embryonic development and wound healing	+4.3
p-PKCα (Ser657)	Phosphorylation of Protein kinase C alpha	+3.9
E-cadherin	Epithelial cadherin	+3.7
PTEN	Phosphatase and tensin homolog	+3.6
TNF-α	Tumor necrosis factor-alpha	+3.0
p-PTEN (Ser380)	Phosphorylation of Phosphatase and tensin	+2.8
p-HGF R/c-MET (Y1003)	Phosphorylation of hepatocyte growth factor receptor	+2.5
p-p90RSK (Ser380)	Activated by Erk1 and 2 in response to many growth factors	+1.9
NF-κB p65	Nuclear factor kappa-light-chain-enhancer of activated B cells	+1.9
CyclinE	Control the progression of cells by activating Cdk	+1.9
cdc2 p34	Cell division control protein 2	+1.9
p-β-Catenin (Ser33/37/Thr41)	Downstream effector in the Wnt pathway	+1.8
Bcl-6	B-cell lymphoma 6 protein	+1.7
p-p70 S6 Kinase (Thr389)	Cell growth and G1 cell cycle progression	+1.7
p-PKC α/βII (Thr638/641)	Phosphorylated at threonine 638 in PKC α and at threonine 641 in PKC β	+1.7
14-3-3β	Relevant to apoptosis, mitogenic signaling and cell-cycle	-5.2
p-p53 (Ser392)	Cellular response to DNA damage	-4.1
Cdk2	Cyclin-dependent kinase 2	-3.8
p53	Transcription activation domain-containing tumor suppressor	-3.5
raf-1	Proto-oncogene serine/threonine-protein kinase 1	-2.7
Cdk6	Cyclin-dependent kinase 6	-2.2
PKCα	Members of the protein kinase C (PKC) family	-2.2
Hsp90	The heat shock protein 90 family	-1.5

Note: "+" fold increase in MSI cells, "-" fold decrease in MSI cells

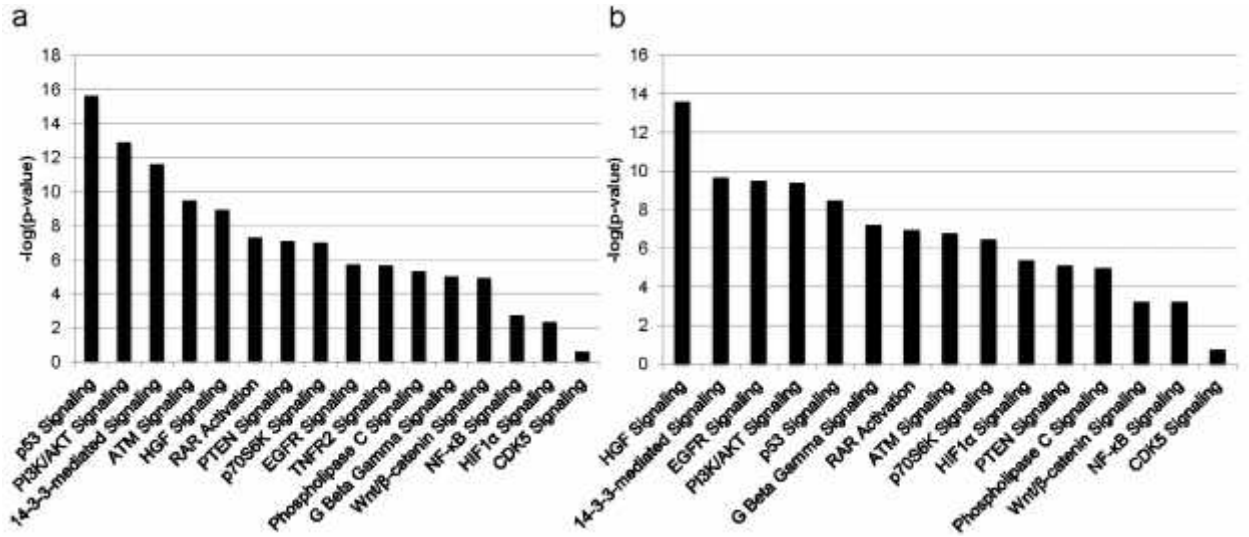

Figure 3. The top canonical pathways altered in (a) MSI cells and (b) KRAS mutant MSS cells as determined by Ingenuity Pathway Analysis (IPA).

Our results show that the p53 pathway plays a critical role in MSI phenotype, since p53 was ranked highest in MSI cells (Figure 3), and was the most important node in the network (Figure 4). p53 responds critically to DNA damage by arresting damaged cells at the G1 phase, thereby allowing damaged DNA to be repaired (14). If the damage cannot be repaired, apoptosis occurs, and the damaged cell is discarded. Both total p53 and phosphorylated p53 (Ser392) were reduced in MSI cells (Table 2). These results suggest that the ability of MSI cells to repair damaged DNA is impaired. In contrast, phosphorylated p53 (Ser392) was increased significantly in K-RAS mutant MSS cells, consistent with the role of functional p53 in MMR competent cells. Phosphorylated HGF (Y1234/1235/Y1003) was increased significantly in MSI cells, suggesting that this pathway is activated. Although increased expression of HGF in MSI tumor cells was reported in the literature (15), activation of this pathway via phosphorylation has not been reported in MSI cells previously. It is worthy to note that phosphorylated HGF (Y1003) was reduced in K-RAS mutant MSS cells, suggesting a different mechanism.

The expression of TNFα and NF-kB was increased in MSI cells, suggesting that an immune pathway was involved. Recent mRNA expression array studies

Table 3. Differential expression of proteins and phosphoproteins between K-ras mutant cells and K-ras wild type cells with MSS

Protein	Description	Ratio (Kras +/-)
TNF-α	Tumor necrosis factor-alpha	+15
p-EGF Receptor (Tyr1068)	Phosphorylation of EGF receptor (EGFR) at Tyr1068	+12.0
ETS1	The v-Ets oncogene isolated from the E26 erythroblastosis virus	+7.8
H-Ras	Transforming protein p21	+7.3
p-CREB (Ser133)	Phospho-Cyclic adenosine monophosphate response element-binding	+3.8
p-PKCα (Ser657)	Phosphorylation of Protein kinase C alpha	+3.4
β-catenin	A key downstream effector in the Wnt signaling pathway	+3.3
p-p53 (Ser392)	Cellular response to DNA damage	+3.1
Hsp90	The heat shock protein 90 family	+2.4
Akt	Plays a critical role in controlling survival and apoptosis	+2.4
Cdk6	Cyclin-dependent kinase 6	+2.3
PKCα	Members of the protein kinase C (PKC) family	+2.1
Cyclin E	Control the progression of cells	+2.0
Survivin	A member of the inhibitor of apoptosis (IAP) family	+2.0
14-3-3β	Relevant to apoptosis, mitogenic signaling and cell-cycle	+1.9
Cdc2 p34	Cell division control protein 2	+1.9
ERK	Extracellular signal-regulated kinases	+1.7
p27	Regulated by a series of cyclin-dependent kinases	+1.7
p-AKT (Ser473)	Plays a critical role in controlling survival and apoptosis	+1.6
NFkB50	Nuclear factor kappa-light-chain-enhancer of activated B cells	-1.6
p14	Be transcribed from an alternate reading frame of the INK4a/ARF	-1.7
Cdk2	Cyclin-dependent kinase 2	-1.7
E-cadherin	Epithelial cadherin	-1.8
PTEN	Phosphatase and tensin homolog	-2.0
Vimentin	Expressed in a highly tissue-specific manner	-2.1
p-HGF R/c-MET (Y1003)	Phosphorylation of hepatocyte growth factor receptor(Y1234/1235)	-2.4
p-PKC α/βII	Phosphorylated at threonine 638 in PKC α and at threonine 641 in PKC β	-3.8
Cdc42	Cell division control protein 42	-5.1
p-Stat3 (Ser727)	A key signaling molecule for many cytokines and growth-factor receptors	-10
p-eIF4B (Ser422)	Phosphorylation of Eukaryotic initiation factor 4B	-10
p-PKCdelta (Thr505)	Phosphorylation of Protein kinase C zeta	-10
p-p38 (Thr180/Tyr182)	Phosphorylation of P38 mitogen-activated protein kinases	-10
Notch4	Neurogenic locus notch homolog protein 4	-10
ATF-1	Cyclic AMP-dependent transcription factor ATF-1	-10
Calretinin	A vitamin D-dependent calcium-binding protein	-10

Note: "+" fold increase in K-ras mutant cells, "-" fold decrease in K-ras mutant cells

showed increased expression of immune response genes and interleukin pathway-related genes (16, 17). It is believed that these pathways are related to the high numbers of tumor- infiltrating lymphocytes histologically observed in MSI tumors. However, our results suggest that MSI colon cancer cells express a high level of immune pathway-related proteins, which may play important roles in tumorigenesis.

The EGFR pathway was involved in both MSI cells and K-RAS mutant MSS cells, ranking number 9 and number 3, respectively (Figure 3). However, the components affected within the EGFR pathway were different (Figure 5). In MSI cells, the EGFR/Raf/ERK canonical pathway was not affected, but its collateral pathways such as p70S6K and p90RSK pathways were activated. In contrast, in K-RAS mutant MSS cells, the EGFR/Raf/ERK canonical pathway was activated, but its collateral pathways such as p70S6K and p90RSK pathways were not affected. In addition, the PKCα pathway was activated in K-RAS mutant MSS cells, but not in MSI cells. These results suggest that the EGFR pathway is important in K-RAS mutant MSS cells, but may not be critical in MSI cells.

In the cell lines investigated, we also compared protein expression with the mRNA levels reported in published data (5). The pattern of mRNA expression between MSI and MSS differs from the pattern of protein and phosphoprotein expression. Among 25 differentially expressed proteins, the expression of only two proteins (increased TNF-α and decreased p53) correlated with their mRNA levels. The discordant results could be due to several factors. The expression microarrays may not contain the genes of interest, especially low abundant regulatory genes. mRNA stability and post-translational modification of proteins could also change the ratio between mRNA and its corresponding protein. In fact, only 48%–64% concordance was observed between mRNA expression and protein level (18). Therefore, direct measurement of proteins, and more importantly, their phosphorylated forms reflects more accurately the function and activity of the pathways.

In conclusion, MSI caused a wide array of dysregulation of many important pathways. The p53 and HGF pathways were affected most significantly. They may play critical roles in the tumorigenesis of MSI CRC. In contrast, activation of the EGFR/RAS/ERK pathway is more important in the K-RAS mutant MSS CRC. Our results suggest that treatment of these two genotypically and phenotypically distinct CRCs should target different pathways. Future studies will focus on understanding the role of these proteins and pathways in MSI CRC.

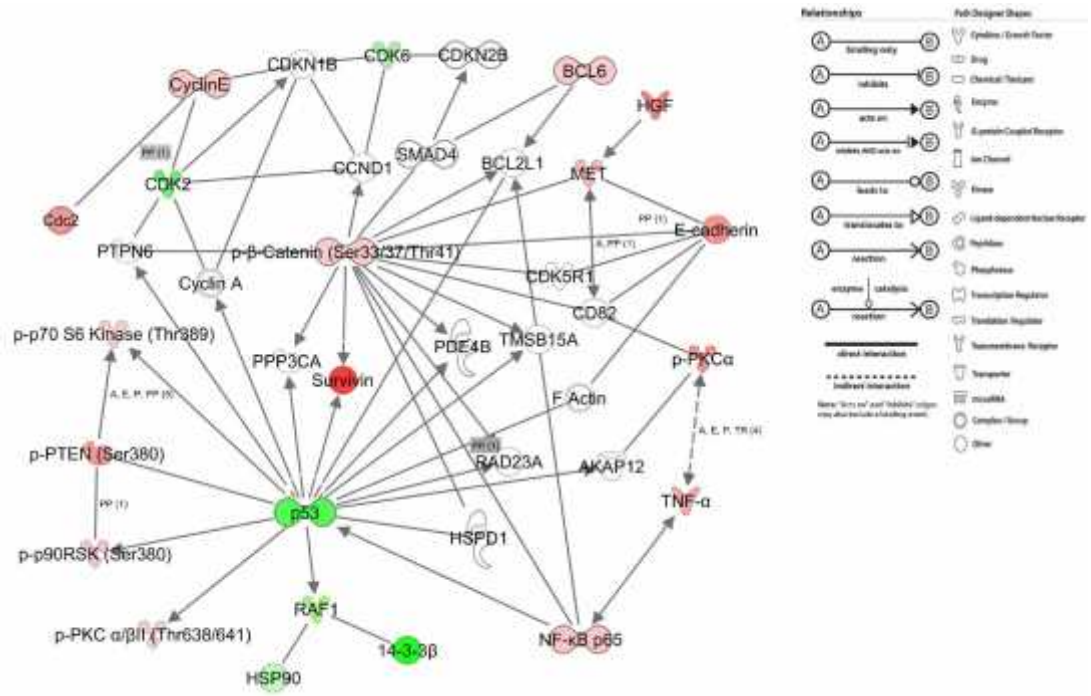

Figure 4. Protein signaling network altered in MSI cells. The network is displayed graphically as nodes (protein) and edges (the biological relationship between the nodes). The nodes are represented using various shapes that represent the functional class of the protein products. The up- and down-regulated proteins in MSI cells shaded in red and green, respectively, according to their fold changes. Proteins without significant difference between MSI and MSS are labeled as yellow. The nodes without color were not assessed in this study but identified by IPA as important nodes involved in the network.

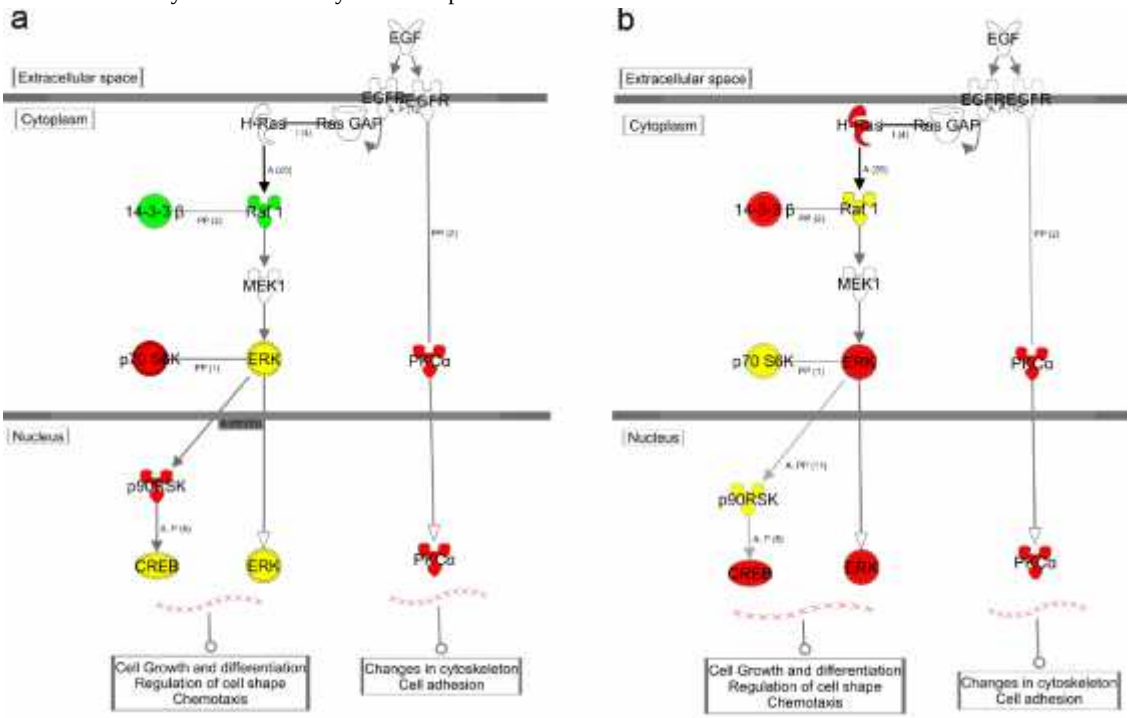

Figure 5. Effect of EGFR and PKCa pathways in (a) MSI cells and (b) KRAS mutant MSS cells. The nodes are represented using various shapes that represent the functional class of the protein. Green indicates down-regulation, red indicates up-regulation and yellow indicates no change. Proteins without color were not assessed in this study. p indicates phosphorylation.

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