Gene Expression Profiling of Microsatellite Unstable and Microsatellite Stable Endometrial Cancers Indicates Distinct Pathways of Aberrant Signaling

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Abstract
Microsatellite instability (MSI) is a molecular phenotype present in ~25% of endometrial cancers. We examined the global gene expression profiles of early-stage endometrioid endometrial cancers with and without the MSI phenotype to test the hypothesis that MSI phenotype may determine a unique molecular signature among otherwise similar cancers. Unsupervised principal component analysis of the expression data from these cases indicated two distinct groupings of cancers based on MSI phenotype. A relatively small number of array features (392) at high statistical value (P < 0.001) were identified that drive the instability signature in these cancers; 109 of these transcripts differed by at least 2-fold. These data identify distinct gene expression profiles for MSI and microsatellite stable (MSS) cancers, which suggest that cancers with MSI develop in part by different mechanisms from their similar stable counterparts. In particular, we found evidence that two members of the secreted frizzled related protein family (SFRP1 and SFRP4) were more frequently down-regulated in MSI cancers as compared with MSS cancers. Down-regulation was accompanied by promoter hypermethylation for SFRP1. SFRP1 was hypermethylated in 8 of 12 MSI cancers whereas only 3 of 16 MSS cancers were methylated. The WNT target fibroblast growth factor 18 was found to be up-regulated in MSI cancers. These data classify histologically similar endometrioid endometrial cancers into two distinct groupings with implications affecting therapy and prevention.

Introduction
Microsatellite instability (MSI) is a common feature of many common epithelial cancers and of those cancers arising in the Lynch (hereditary nonpolyposis colorectal cancer) cancer syndrome. The phenotype is diagnostic of an underlying defect in one or more DNA mismatch repair genes either by mutation or by epigenetic silencing. The exact mechanism by which cancer arises from this defect remains controversial. One widely accepted possibility is that an increased mutation rate due to mismatch repair deficiency leads to an accumulation of mutations in genes normally important in the control of cancer. Indeed, substantial correlative evidence exists for this idea, particularly in colon carcinoma where tumor suppressor or antiapoptotic genes containing hypermutable repeat tracts located within the coding region of the gene are frequently mutated in MSI cancers. This is best exemplified for the TGFBR2 gene, which is mutated in a hypermutable (A)10 tract in the majority of colorectal cancers with MSI (1). However, this repeat tract and others are not universally mutated in other MSI epithelial cancers including endometrial cancers (2, 3). Cells defective in mismatch repair also show defects in cellular signaling mechanisms that normally can lead to apoptosis or growth arrest (4). Additionally, data indicate various survival advantages of mismatch repair–negative cells under adverse conditions. Specifically, defective mismatch repair in yeast can lead to increased survival in the absence of telomerase activity, and human mismatch repair–negative cells are more likely to survive in hypoxic and low-pH environments (5, 6). Cells defective in mismatch repair often fail to transmit proapoptotic signals following various cellular challenges including alkylating agents, DNA cross-linking agents, as well as oxidative stresses, suggesting the additional possibility of differential responses to chemopreventive or therapeutic agents (4, 7). This effect may be mediated by transcriptional inhibition dependent on MLH1 and p53 (8).

Approximately 20% to 25% of nonfamilial endometrial cancers have the MSI phenotype and it occurs almost exclusively in cancers of endometrioid histology (9). Although many of these cancers are generally curable by surgery alone, this disease is the most frequent gynecologic malignancy in western societies and is associated with high costs in terms of morbidity. Studies suggest that microsatellite unstable (MSI) and microsatellite stable (MSS) cancers have different clinical outcomes (10–13). In our study of stage I through stage IV endometrial cancers, MSI cancers were associated with other favorable prognostic indicators (14). However, when stage I and stage II endometrial cancers are examined separately, MSI status is associated with recurrence and with poorly differentiated cancers, indicating that these cancers are more likely to require additional clinical attention (15). More extensive clinical outcomes studies on endometrial cancer are necessary to resolve these inconsistencies.

Endometrial cancer is a cancer of which incidence and mortality are affected by association with high body mass index, suggesting that the incidence and mortality of this cancer may dramatically increase along with increasing trends of weight gain in western societies (16, 17). The clear association with high body mass index also suggests that many endometrial cancers may be readily preventable. Preventive or therapeutic strategies may depend, in part, on underlying genetic or epigenetic differences in particular...
cancers or individuals. It is imperative to understand the underlying biology of endometrial cancers and to develop effective preventive and nonsurgical therapeutic strategies for this disease.

Like many other cancers, endometrial cancers are thought to develop in part due to accumulation of defects in cellular growth control pathways. In addition to the MSI phenotype, many endometrial cancers contain mutations of the PTEN and TP53 tumor suppressor genes, mutagenic activation of the KRAS2 oncogene, and mutagenic stabilization of β-catenin (CTNNB1; ref. 18). None of these events occur in all endometrial cancers and the frequency of these alterations differs in various histologic types (19). We previously showed that gene expression profiling can also clearly distinguish between the different histologic types of endometrial cancer (20). In this study, we sought to determine whether the MSI phenotype in early-stage endometrial cancers is associated with characteristic patterns of gene expression that could be defined using microarrays. These profiles may be useful in determining future treatment options tailored to individual cancers.

Materials and Methods

Clinical specimens. Flash-frozen endometrial cancers were obtained from patients undergoing hysterectomy at Duke University Medical Center. None of the patients had received preoperative chemotherapy or radiation. In addition, samples of histologically normal endometrium were obtained from patients undergoing hysterectomy for benign gynecologic diseases. Tissues were obtained at Duke with Institutional Review Board–approved informed consent and this study was approved by the National Cancer Institute Institutional Review Board. Tissue samples were subjected to RNA isolation using TRIzol and an additional purification using the Rneasy Kit from Qiagen, Valencia, CA following the recommendation of the manufacturer. Following isolation of RNA, the integrity of each RNA sample was verified by denaturing gel electrophoresis. Genomic DNA was isolated from cancer and noncancer tissue following proteinase K digestion and phenol/chloroform extraction.

Microsatellite instability analysis. Normal and genomic DNAs from each patient were examined using the National Cancer Institute/Bethesda panel of microsatellite markers (BAT25, BAT26, D2S123, DSS346, and D17S250) using a modification of the fluorescent protocol developed by Berg et al. (21). Briefly, markers were amplified separately but electrophoresed simultaneously on an ABI 3700 capillary sequence detection system. Peaks were analyzed using genescan software. Cancers were either MSI-high (MSI-H) when at least two of the five markers exhibited differences in allele size as compared with the patient’s matched normal DNA control or microsatellite stable (MSS) when no marker exhibited change. None of the cancers exhibited the MSI-low (MSI-L) phenotype where only one of five markers exhibits differences. Cancers in this article are therefore described as MSS or MSI.

Gene expression array. We studied gene expressions using Affymetrix Human Genome-U133A and HG-U133B Gene Chip expression arrays (45,000 gene transcripts covering 28,473 UniGene clusters). Five micrograms of total RNA from each sample were labeled using the bioarray high yield transcript kit according to the conditions of the manufacturers (ENZO Life Sciences, Inc., Farmingdale, NY). Labeled RNAs were hybridized and washed according to the recommendations of the manufacturer (Affymetrix, Inc., Santa Clara, CA). Microarray Suite 5.0 software (Affymetrix, 2001) was used for initial gene expression data analysis. All arrays were normalized to a trimmed mean transcript signal level of 500 counts. The genes associated with “absent” signal detection calls in 70% of arrays both in MSI and MSS samples were excluded from further analysis. The signal values of the remaining 22,378 genes were used for statistical analysis. All the statistical calculations were done on the logarithmic values of signals.

Principal component analysis. Principal component analysis minimizing correlations were done on 17,780 gene expressions having present calls in at least 50% of the arrays. A projection was made on the largest three principal components accounting for 26% of the total variance (computed using software Partekpro 5.0, Partek, Inc., St. Charles, MO).

Differentially expressed genes. Parametric Student’s t tests indicated 392 gene transcripts were differentially expressed between MSS and MSI at P < 0.001. Multivariate permutation test based on 10,000 permutations estimated about 3% false discoveries among these transcripts and the probability of getting at least 392 genes significant by chance if there are no real differences between the classes as 0.0001 (BRB Array tools ver. 3.0c, Richard Simon, Amy Peng, Biometric research branch, National Cancer Institute, NIH; http://linus.nci.nih.gov/BRB-ArrayTools.html).

Methylation-specific PCR. Specific primers for unmethylated and methylated DNA following sodium bisulfite conversion of DNA were designed within the CpG island of secreted frizzled related protein 1 (SFRP1). Primers for unmethylated PCR were U2F-gttttgtgttaggttgtgtgt and U2R-acaacaacccecccttacta. Primers for the methylated PCR were M2F-gtttggctagacgagcccgc and M2R-gcacgacccctcctgacg. PCR assays were verified by analysis of endometrial carcinoma cell lines, HEC-1-A, containing a methylated promoter and no SFRP1 transcript expression, and KLE, which expresses abundant SFRP1 and exhibits no promoter methylation. Reverse transcription-PCR primers for SFRP1 were 87AF-gaggttgaatctgaggccatca and 1061R-tggtgcagggagccagctca.

Hierarchical clustering. The set of 109 array features having 2-fold differential expression (P < 0.001) between MSI and MSS were clustered by the similarity of their expression profiles. Hierarchical clustering was done on logarithmic values of expressions using 1 - p as distance metric, where p is the correlation coefficient of any two gene expressions (22). The expression data were shown relative to average gene expression.

Results and Discussion

We first analyzed 24 endometrial cancers of similar stage, grade, and endometrioid histology (and their matching normal DNAs) for the MSI phenotype utilizing the Bethesda panel of simple repeat markers (23). We found that 9 of 24 cancers exhibited the MSI-H phenotype wherein at least two of the five markers exhibited additional alleles as compared with the matched normal sample. We found no evidence for the MSI-L phenotype in this group of cancers. The remaining 15 cancers did not show evidence of MSI and were designated MSS. To explore the global gene expression profiles of these samples, we did gene expression array analysis on all 24 samples using Affymetrix Hu133A and Hu133B chips. Initially, we examined the possibility of underlying expression profiles by examining the data obtained from these 24 endometrial cancers using unsupervised principal component analysis. Principal component analysis is a linear transformation of the data to study variances by minimizing the covariances or correlations. This can be used for dimension reduction when most of the variance is accounted by a few principal components. The resulting data will separate samples with like expression patterns. Principal component analysis on the expression data from this set of endometrial cancers revealed distinctively separated groups for MSI and MSS endometrial cancers, indicating consistent expression changes for each type of sample when plotted in three dimensions (Fig. 1). These groupings were quite distinct, indicating that a consistent set of gene expression differences were driving this separation.

To determine the genes responsible for these groupings, we examined those transcripts that are significantly different between the MSI and MSS groups. We applied stringent statistical analysis and identified 392 transcripts at P < 0.001. Experimental and biological validation is an important component of any global transcript analysis. Importantly, we identified the hMLH1 gene as one of the distinguishing transcripts. Epigenetic silencing of the hMLH1 gene is the principal defect identified in nonfamilial endometrial cancers with the MSI phenotype (24–26). In addition,
another transcript identified was the KIAA0766 gene, also known as EPM2AIP1, located and transcribed from the opposite strand from MLH1. Both these genes share the same CpG island that is hypermethylated and silenced in many MSI cancers and both were found down-regulated in MSI cancers (24, 27). These two transcripts provide an important internal control lending (biological) validity to our results. In addition to identifying these important transcripts, we also show that hierarchical clustering also separated the cancers into two main groupings. A portion of these data are depicted in Fig. 2 with clear differences in some 109 array features exhibiting at least 2-fold difference in gene expression at high statistical significance \((P < 0.001)\), and 537 features at least 1.5-fold at \(P < 0.005\) (Supplementary data). The expression of these transcripts was also compared with the average expression of each in eight normal endometria.

The expression of a subset of these differentially transcribed was analyzed by real-time PCR to further validate our array results. Real-time PCR confirmed the differential expression of polycystic kidney and hepatic disease like 1 (PKHD1L1), heat shock 22 kDa protein 8 (HSP80), secreted frizzled related protein 4 (SFRP4), and fibroblast growth factor 18 (FGF18) in these cancers including three additional cases of each of MSI and MSS cancers, lending further confirmation to our data set (Fig. 3). These genes may also contribute to the carcinogenic process in these cancers.

We first examined the expression of the PKHD1L1 gene to validate our array findings. This transcript, also known as DKEF2p586C1021, displayed the greatest fold change between MSS and MSI cancers based on our array data (Fig. 2). Real-time PCR confirmed this change, indicating a more than 10-fold increase on average as compared with MSI-positive cancers (Fig. 3D). This gene resides on chromosomal location 8q23 and encompasses a region of over 160 kb and contains at least 78 exons. The predicted protein has the features of a cell membrane receptor but its function is unknown (28). Interestingly, the 8q22-24 region is frequently subjected to increased copy number in many endometrial cancers, in particular those endometrial cancers without MSI (29, 30), making PKHD1L1 a possible gene candidate for this region. We also analyzed the expression of PKHD1L1 in laser-captured epithelial, stroma, and myometrial cells from normal endometria and found expression primarily in the epithelial cells (data not shown).

We also examined the levels of two negative regulators of WNT signaling, SFRP1 and SFRP4. Both transcripts were down-regulated as determined by the microarray. SFRP4 was previously reported as a stromal specific gene involved in the negative regulation of WNT signals (31). We noted a marked decrease in SFRP4 levels in MSI cancers as compared with normal endometria and with MSS cancers, and confirmed this by real-time PCR analysis (Fig. 3). Furthermore, we examined the tissue-specific nature of SFRP4 in laser-captured normal endometria and found transcripts in epithelial, stromal, and myometrial cell types (data not shown). In addition to SFRP4, the related family member SFRP1 gene was also more down-regulated in MSI cancers.

We examined the relationship of down-regulation of this gene to promoter methylation status. SFRP1 and another secreted frizzled related transcripts have been previously identified in an epigenetic screen to identify transcripts silenced by hypermethylation in colon carcinoma (32). First, we examined the expression of SFRP1 in a small panel of endometrial carcinoma cell lines. The KLE and HEC-59 cells expressed readily detectable transcripts, whereas SFRP1 transcript was not detectable in HEC-1-A or HOUA cells (data not shown). HEC-1-A cells were treated with the demethylating agent 5-aza-2-deoxycytidine and examined for reexpression of SFRP1. SFRP1 expression was readily detected in 5-aza-2-deoxycytidine-treated cells, indicating some level of epigenetic control of this locus either directly or indirectly. We next examined the DNA sequence of the 5’ region of the gene and designed primers that could distinguish between methylated and nonmethylated regions following sodium bisulfite treatment. Methylation of the promoter was found to be coincident with a lack of expression of SFRP1 in these cell culture models. SFRP1-expressing cells contained unmethylated alleles of SFRP1 whereas nonexpressing cells were methylated. We next applied this assay to the cancers in our study to determine if promoter hypermethylation might be the mechanism for the observed decreased expression of SFRP1 more frequently noted in MSI cancers. We detected methylated alleles in 8 of 12 MSI cancers whereas we detected methylation in only 2 of 16 MSS cancers \((P = 0.0031)\; (\text{Fig. \, 4})\).

In this report, we found that FGF18 transcripts were largely undetected in normal endometria and in most MSS cancers but were up-regulated in MSI cancers. The role of FGF18 is largely unexplored in carcinogenesis. Other FGF family members possess a variety of protumorigenic characteristics and a recent report indicates that FGF18 promotes colon tumorigenesis and is also a direct target of an activated WNT pathway (33). Aberrant WNT signaling has been implicated in endometrial carcinogenesis and a subset of endometrial cancers contain activating mutations of β-catenin (CTNNB1) or epigenetic silencing of APC (34–36). However, the downstream transcriptional targets of this pathway have not been explored to any extent in the endometrium.
These findings indicate significant differences in the aspects of WNT pathways between MSI and MSS cancers. Our data is consistent with the conclusion in a previous report that APC promoter methylation occurs more frequently in MSI than in non-MSI endometrial cancers (34). The APC, FGF18, SFRP1, and SFRP4 data taken together support the idea that MSI endometrial cancers may exhibit a more active WNT pathway than do MSS endometrial cancers. The necessity of the cancer cell to inactivate

Figure 2. Gene expression differences that distinguish MSI-H and MSS endometrial cancers. Hierarchical clustering of 109 transcripts differentially expressed (P < 0.001) with a threshold of at least 2-fold. Red, up-regulation; green, down-regulation.
multiple negative regulators of the same pathway is curious and may reflect our incomplete knowledge of the subtleties of the WNT signaling pathways or may indicate that the epigenetic down-regulations of these genes are less complete than perhaps mutational inactivation. An interesting observation is that inactivation of SFRP family members occurs even in colon cancers with mutation in downstream members of this pathway, such as APC in colorectal cancer (37). APC mutation has not been described for endometrial carcinoma; however, some contain activating mutation of CTNNB1. Exploration of the intricacies of WNT signaling regarding the various WNT regulators in cancer may be facilitated by our findings in future studies.

A large body of literature support a role for unopposed estrogens in the development of endometrioid endometrial carcinomas. Interestingly, the H11 gene, which is under the tight control of estrogens, was reduced in MSI cancers (Figs. 2 and 3C). H11 expression is dependent on the presence of estrogen receptor in breast cancers and is a marker for estrogen receptor–positive cases (38). Although the function of H11 is unknown, it may be a biomarker for estrogen receptor cancers and its reduced expression in MSI cases may be indicative of reduced estrogenic activity in this subset. Relative levels of H11 were reduced in both MSI and MSS cancers as compared with normal endometria; however, this observation was more pronounced in MSI cancers. The data support a model in which estrogens are less important in the development of MSI cancers, which may be driven by other factors (perhaps WNT).

To further examine the stringency of our data for binary class comparison on the MSS and MSI groups, we tested the compound covariate class predictor. This class prediction was done using the
392 genes that distinguished MSS and MSI by two-sample t test ($P < 0.001$; ref. 39). The prediction was done by leaving one sample out at a time for cross-validation and using all other samples for classification. Based on 10,000 random permutations, the compound covariate predictor was correct for 92% of the arrays with a $P$ value of 0.001. The validity of this set of genes was further verified using three other classifiers including support vector machine, linear discriminant analysis, and the nearest centroid classifier (BRB Array tools ver. 3.0c, Richard Simon, Amy Peng, Biometric research branch, National Cancer Institute, NIH; http://linus.nci.nih.gov/BRB-ArrayTools.html), which also correctly classified 88% to 92% of the MSI and MSS arrays, further validating the data.

It is perhaps somewhat surprising that such distinct groupings were determined from our unsupervised analysis because the underlying defect is in the DNA mismatch repair system and not in a transcription factor(s), which might be anticipated to give such expression profiles. For example, estrogen receptor status defines distinct gene expression patterns in breast cancers (40). However, this phenomenon is not without precedent. During the preparation of our manuscript, microarray data that discriminate MSI-H, MSS, and MSI-L groups have been identified in colorectal cancers as distinct global molecular phenotypes (41). Similar to our data obtained in the endometrium, colon cancers designated MSI-H, MSS, and MSI-L groups have been identified in colorectal cancers as distinct global molecular phenotypes (41). Similar to our data obtained in the endometrium, colon cancers designated MSI-H, MSS, and MSI-L groups were separated into distinct groupings by principal component analysis. In addition, this report was able to further identify the MSI-L group as a valid entity in colorectal carcinomas. We have found no evidence for the MSI-L group in this panel of endometrial cancers and our principal component analysis supports this in that cases were either MSI-H or MSS. However, it is possible that a MSI-L group is rare or may exist for endometrial carcinomas in more advanced cases or in cases of nonendometrioid histology.

We addressed the issue of whether the same set of genes were driving the molecular expression profiles of colon and endometrial MSI-H cancers. Mori et al. reported 20 genes in addition to MLH1 as transcripts that most strongly drove the MSI-H phenotype in colorectal cancer. We therefore examined those 20 genes (and also MLH1) which were present on the Affymetrix array chips and found that none were present on our list of differentially expressed genes even when we examined our data at a reduced statistical stringency ($P < 0.005$). However, MLH1 was identified in both studies and seemed to be the only consistently observed feature.

It is possible that the strong separation of MSI and MSS cancers using microarrays is based on another underlying defect that cosegregates with the MSI phenotype or MLH1 defect. Subsequent analysis of the MSI-positive cancers in our panel indicated that they all exhibited hypermethylation of the MLH1 gene promoter whereas this gene was not methylated in any of the MSS group (data not shown). Perhaps the profile is indicative of an undescribed phenotype related to global methylation phenotype? However, promoter methylation analysis of other genes on this same group of cancers does not support the general contention that MSI cancers strictly fall into a “methylator phenotype group”.

Many cancers in the MSS group also contain methylated promoters and MSI cancers have unmethylated ones. Additionally, it is not clear whether an endometrial cancer with a mutation (rather than epigenetic silencing) of MLH1 or of another mismatch repair gene (i.e., MSH2 or MSH6) would exhibit a gene expression profile similar to the MSI cases described here or of a separate category.

Our data suggest that the MSI and MSS early-stage endometrioid endometrial cancers have distinct patterns of gene expression. The gene expression data related to this phenotype can also be interpreted to support the idea that the carcinogenic program in MSI cancers likely extends beyond the simple hypothesis that these DNA repair–deficient cancers are solely the result of an accumulation of mutations in growth control genes. Importantly, we identified new molecular targets which may be useful in understanding the etiology of this cancer type. A better understanding of the molecular pathogenesis of MSI and MSS endometrial cancers has the potential to facilitate improvements in the prevention and treatment of these cancers.

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