Prediction of Lymph Node Metastasis in Patients with Endometrioid Endometrial Cancer Using Expression Microarray

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Abstract

Purpose: To characterize the gene expression profiles of endometrioid endometrial cancers associated with lymph node metastasis in an effort to identify genes associated with metastatic spread.

Experimental Design: Tumors from 41 patients with endometrioid endometrial cancer grossly confined to the uterine cavity were evaluated. Positive lymph nodes were noted in 12 of 41 patients. RNA was analyzed for gene expression using the Affymetrix HG133A and HG133B GeneChip set, representing 45,000 array features covering >28,000 UniGene clusters. Data analysis was done using multidimensional scaling, binary comparison, and hierarchical clustering. Gene expression for several differentially expressed genes was examined using quantitative PCR.

Results: Gene expression data was obtained from 30,964 genes that were detected in at least 5% of the cases. Supervised analysis of node-positive versus node-negative cases indicated that 450 genes were significantly differentially expressed between the two classes at \( P < 0.005 \), 81 of which were differentially expressed by at least 2-fold at \( P < 0.005 \). Overexpressed genes included two cell cycle checkpoint genes, CDC2 and MAD2L1, which have previously been described in association with lymph node metastasis in other cancer types. The ZIC2 zinc finger gene was overexpressed in endometrial cancers with positive nodes versus those with negative nodes.

Conclusion: Gene expression profiling of the primary tumors in patients with endometrioid endometrial cancers seems promising for identifying genes associated with lymph node metastasis. Future studies should address whether the status of nodal metastasis can be determined from the expression profiles of preoperative tissue specimens.

Most endometrioid endometrial cancers are confined to the uterus at diagnosis, but ~15% of apparent early stage cases have occult metastases and are destined to develop recurrence. Surgical staging, including selective lymphadenectomy, facilitates identification of patients who have metastatic disease. The decision to perform selective lymphadenectomy generally is made intraoperatively based on known risk factors such as histologic grade and depth of myometrial invasion. The risk of pelvic lymph node metastases is >10% in patients with cancers that are poorly differentiated or that invade the outer half of the myometrium (1). Surgical staging facilitates individualization of adjuvant therapy by identifying those at highest risk of recurrence. In addition, there is some evidence that surgical resection of nodal metastases may have a therapeutic benefit (2).

Although several risk factors for nodal metastasis have been identified, they have a low positive predictive value in clinical practice. For example, less than half of patients with poorly differentiated, deeply invasive endometrial cancers have lymph node metastases. In addition, preoperative radiologic testing using ultrasound (3), computed tomography (4), or magnetic resonance imaging (5) is suboptimal in identifying patients with pelvic or paraaortic nodal metastases. These radiologic studies are much more accurate in the identification of patients with a low rather than high likelihood of nodal metastasis. Elevated CA 125 levels are associated with lymph node metastasis (6), but abnormal values occur most often in those with nonendometrioid cancers (7, 8) in which surgical staging is standard practice irrespective of other features. Identification of an accurate test predictive of node metastasis in endometrial cancer would have potential clinical applications. Endometrial cancer patients with a preoperative test predictive of node metastasis could be referred to a subspecialty gynecologic

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oncologist, whereas patients with a low likelihood of node metastasis could be managed by the general obstetrician/gynecologist. Improvements in referral patterns would be a more effective use of health care resources.

Many studies have examined the relationship between specific molecular alterations and endometrial cancer behavior; however, few have specifically focused on the association of molecular alterations with lymph node metastasis (9–12). Although p53 mutation, as determined by protein overexpression/stabilization in endometrial cancer, was shown by some investigators to be associated with extrauterine spread in univariate regression (9, 10), this relationship has not been consistently confirmed (11), particularly in studies that employed multivariate modeling (12). Likewise, overexpression of BCL-2 (10–12) or HER-2/neu (10, 11) and/or PTEN mutation (13), are not associated with lymph node involvement in endometrial cancer.

Microarrays allow global analysis of gene expression and provide the opportunity to identify patterns that underlie biological differences between cancers. In this study, we examined whether there are significant differences in gene expression patterns between endometrial cancers that are solely confined to the uterus versus those with concurrent lymph node metastases. Identification of unique gene expression patterns associated with endometrial cancers associated with node metastasis would justify more definitive studies evaluating the predictability of preoperative gene expression in identifying endometrial cancer patients with lymphatic involvement.

Materials and Methods

Tissue specimens. A query of >400 endometrial cancers included in the Duke Gynecologic Tissue Bank was done to identify cases of endometrioid endometrial cancer that seemed to be confined to the uterus but ultimately were found to have metastasized to the pelvic and/or aortic lymph nodes. Only tissue specimens containing >50% uterus but ultimately were found to have metastasized to the pelvic and/or aortic lymph nodes. Only tissue specimens containing >50% cancer were chosen for the analysis. Twelve cases of stage IIIC endometrial cancer were found that met these criteria. Twenty-nine cases of stage I or II endometrioid endometrial cancer with negative nodes on surgical staging were selected for comparison. Flash-frozen endometrial cancer specimens were obtained from the primary tumors of these 41 patients. All tissues and data were collected under an Institutional Review Board–approved protocol at Duke University Medical Center. Tissue samples were subjected to RNA isolation using Trizol followed by an additional level of purification with the RNeasy Kit (Qiagen, Valencia, CA). The integrity of each of the RNA samples was confirmed using gel electrophoresis.

Gene expression analysis. The Affymetrix (Santa Clara, CA) HG-U133A and HG-U133B (45,000 gene transcripts covering 28473 UniGene clusters) GeneChip System was used to analyze the global gene expression for each of the endometrioid specimens. Total RNA (5 μg) from each sample was labeled with a high-yield transcript labeling kit and labeled RNAs were hybridized, washed, and scanned according to the manufacturer’s specifications (Affymetrix, Inc.). The Microarray Suite 5.0 software (Affymetrix) was used to process images and estimate transcript expression levels. Total intensity normalization was applied by normalizing all arrays to an average signal level of 500 counts. Signal detection calls were used for initial screening of expression data. All the statistical calculations were done on the logarithmic values of signals.

Data analysis. Unsupervised data analysis was done using multidimensional scaling (computed by Partek Pro Discover Software Build 6, Partek, Inc., St. Charles, MO) using 1-Pearson correlation as distance metric of 18,168 genes detected in at least 50% of the samples. The expression data were shown relative to average gene expression and facilitate clustering of cases according to similarities in global gene expression illustrated in three-dimensional space. Supervised analysis was done using hierarchical clustering and binary class comparison and prediction. We chose to use a 95% absent call criteria in order to maximize the likelihood of detecting genes that were expressed at a low frequency across samples.

Real-time PCR. The relative expression of genes chosen for validation was determined by quantitative PCR using FAM-labeled TagMan Gene Expression Assays purchased from Applied Biosystems (Foster City, CA) with VIC-labeled (β-actin [4326315E] as reference. For each group, four targets were independently assessed: CDC2 (Hs00176469_m1), MAD2L1 (Hs00829154_g1), TOB2 (Hs00249392_s1), and ZIC2 (Hs00600845_m1). Samples were run in separate tubes on the ABI Prism 7700 Sequence Detection System according to the manufacturer’s suggested protocols. The relative quantitation, using the comparative Ct method, was calculated for each sample. The weighed average of the mean ratios of each group was presented with the SE of mean values as error bars.

Results

The characteristics of the patients in the node-positive and node-negative groups in our study were different in terms of stage and grade. All 12 of the node-positive patients were stage IIIC, whereas 25 node-negative patients were stage I (7 patients stage IA, 11 patients stage IB, and 7 patients stage IC), and 4 were stage II (3 patients IIA and one patient at stage IIB). As a first step in evaluating the hypothesis that gene expression profiles present in the primary cancer could predict the presence or absence of node metastasis, we examined the entire cancer data set using unsupervised analysis. This analysis can serve to distinguish similar and dissimilar groupings of cancers based on global gene expression, however, more subtle differences between groups may not be apparent. For the endometrioid cancers in this study, unsupervised analysis using principle component analysis revealed only marginal separation and failed to indicate a discreet clustering of expression profiles from cases of node-positive versus node-negative endometrioid endometrial cancer (Fig. 1). These data indicate that node status is not the principal delineating feature of these cancers.

In order to assess if a group of genes distinguishes cases with positive lymph nodes (n = 12) from those without lymph node metastasis (n = 29), we did a supervised analysis of the expression data. This analysis indicated that 450 array features were differentially expressed between node-positive and node-negative cases by parametric Student’s t test (P < 0.005). We further examined the stringency of this list by estimating the number of false discoveries, which might be present and found this to be <10%. In addition, a multivariate permutations test based on 10,000 permutations indicated the probability of ascertaining at least 450 genes as significantly differentially expressed by chance if there were no real differences between the classes was 0.02 (computed using software BRB Array tools version 3.0c, Richard Simon, Amy Peng, Biometric Research Branch, National Cancer Institute, NIH, http://linus.nci.nih.gov/BRB-ArrayTools.html).

We next evaluated transcripts using hierarchical clustering methods and chose to examine a subset of the differentially expressed genes choosing those that varied by at least 2-fold at
P < 0.005. Eighty-one transcripts clustered according to the similarity of expression profile. The 25 highest and 25 lowest genes differentially expressed genes (at least 2-fold) at P < 0.005 are shown in Fig. 2. Because our analysis indicated as much as 10% false positives, we chose to examine several transcripts by an alternate gene expression method in order to validate our statistical approaches. Real-time PCR confirmed the differential expression patterns of ZIC2, CDC2, and MAD2L1, validating our array findings (Fig. 3).

Because many of the endometrial cancer cases that involved lymph node metastasis were from patients with grade 3 tumors, we analyzed our data to confirm that gene expression associated with lymph node metastasis was not a reflection of the gene expression associated with grade. In this analysis, we did a binary comparison (P < 0.005) of grade 1 (n = 16) versus grade 3 (n = 15) tumors using cases, irrespective of stage of disease. The cases of G2 (n = 5) were excluded because we were attempting to create a more discriminatory analysis of grade and did not acknowledge whether G2 tumors would be more like G1 or G3 from the perspective of gene expression. The gene list from the node-negative versus node-positive comparison was cross-referenced with the gene list from the grade 1 versus grade 3 comparison in an effort to identify genes common to both lists. It was assumed that if the gene expression associated with grade and node metastasis were similar, there should be differentially expressed genes common to both comparisons. An analysis of these two gene lists revealed no significant overlap (included as Supplemental Data).

The presence of multiple gene transcripts involved in mitotic cell division as exemplified by CDC2 and MAD2L1 was readily apparent in gross examination of the significant gene list. We further analyzed the significant genes that characterized node-positive primary cancers by examining the list for overrepresented gene ontologies. Using the Expression Analysis Systematic Explorer software, we found increased mitotic cell

Discussion

Global expression analysis seems to be promising in identifying genes associated with specific clinical profiles in patients with endometrial cancer (14, 15). The intent of our study was to identify specific genes that are associated with lymph node metastasis. Identification of gene expression patterns associated with metastasis in early stage endometrial cancer may facilitate future studies aimed at the prediction of metastasis.

In the class comparison of node-negative and node-positive cases, we found that the ZIC2 gene was significantly overexpressed in endometrial cancer cases with node metastasis. ZIC2 is one of three homologues of the Drosophila Odd paired gene, the functions of which are poorly understood. The ZIC genes encode zinc finger proteins that are critical in the development of the central nervous system (16) and mutations in ZIC2 can lead to holoprosencephaly (17). Aberrant expression of ZIC2 has also been observed in 90% of medulloblastomas (18, 19), and in >60% of small cell lung cancers (20). Examination of the literature and CGAP public expression data indicate support for our findings; no expressed sequence tags for ZIC2 were found in normal uterine libraries nor was ZIC2 found expressed in the uterus in a study evaluating its expression in several human tissues (20). These data suggest that ZIC2 may be an attractive marker for node metastasis in endometrioid endometrial cancer based on its relative absence in normal endometria and high percentage of expression in node-positive primary cancers. The data linking ZIC2 to other types of cancers is limited, but suggests that expression may be associated with cancers of the bladder, colon and breast (20). Evidence has suggested that ZIC proteins might act as transcriptional activators in modulating cellular processes (21). In a recent study, investigators used protein/DNA array to identify ZIC2 as one of several transcription factors that bind to the β-catenin gene (CTNNB1; ref. 22). Although the overall expression of β-catenin protein is dependent on multiple transcriptional and posttranscriptional factors (23), overexpression has been observed in up to 38% of endometrial cancer cases (24). In our analysis, we did not observe a differential expression in CTNNB1 that was concurrent with ZIC2. An association between lymph node metastasis and overexpression of β-catenin protein that might result from transcriptional activation has not been reported. However, mutations in CTNNB1 are more frequent in endometrioid endometrial cancers lacking lymph node metastasis compared to those cases with lymph node metastasis (25). Our findings suggest that ZIC2 overexpression may influence node metastasis through mechanisms other than CTNNB1 transcriptional activation.

Review of the differentially expressed genes in the node-positive versus node-negative comparison also revealed overexpression of TOB2 (an antiproliferative factor; ref. 26) in node-negative cases. These findings were apparent for only one of two probe sets represented on the Affymetrix GeneChips.
The other primer set for the array for TOB2 did not reveal differential expression. Validation of the array findings using quantitative PCR did not confirm differential expression between the node-positive and node-negative groups and was consistent with the probe set on the microarray chip that showed similarity between groups. Because of the possibility of differentially expressed splice variants of this gene, more careful examination of RNA isoforms of TOB2 in relation to node metastasis in endometrial cancer is warranted.

Cell cycle checkpoint genes are important for cell cycle regulation and abnormal expression can be associated with carcinogenesis. In our analysis, we identified overexpression of CDC2 and MAD2L1 in endometrial cancer cases with lymph node metastasis. CDC2 is a cyclin checkpoint gene that regulates cell cycle progression through the G1-S and G2-M transitions and involves modulation by both the retinoblastoma and p53 tumor suppressor genes. Increased expression of CDC2 has been associated with leiomyosarcoma of the uterus (27), neoplastic squamous epithelium of the cervix (28), and hepatocellular carcinoma (29). Few studies have evaluated overexpression of CDC2 in association with clinical and pathologic factors. However, recent evidence indicates that overexpression of CDC2 is associated with lymph node metastasis in both breast cancer (30) and colon cancer (31). MAD2L1 (mitotic arrest defect-2L1) was also noted to be significantly overexpressed in endometrial cancer cases with nodal metastasis. MAD2L1 is a member of a group of mitotic checkpoint genes responsible for the maintenance of metaphase arrest. Defects in mitotic checkpoint control are thought to promote chromosomal instability and aneuploidy.

There have been several studies using comparative genomic hybridization to address DNA gain and loss in endometrial cancers. At least one of these studies compared various clinical features including lymph node status with certain chromosomal aberrations (32). Suehiro et al. indicated a significant increase in 8q22-23 and 8q24-qter DNA copy number gains in node-positive endometrial cancers (32). We did an examination of the 450 array features identified in our study as differentially expressed between node-positive and node-negative for any genes located on chromosome regions 8q22-23 and 8q24-qter. Four transcripts were present including three located on 8q24.3. However, all three of these showed down-regulation in node-positive as compared with node-negative for any genes located on chromosome regions 8q22-23 and 8q24-qter. Four transcripts were present including three located on 8q24.3. However, all three of these showed down-regulation in node-positive as compared with node-negative for any genes located on chromosome regions 8q22-23 and 8q24-qter. Four transcripts were present including three located on 8q24.3. However, all three of these showed down-regulation in node-positive as compared with node-negative for any genes located on chromosome regions 8q22-23 and 8q24-qter. Four transcripts were present including three located on 8q24.3. However, all three of these showed down-regulation in node-positive as compared with node-negative for any genes located on chromosome regions 8q22-23 and 8q24-qter. Four transcripts were present including three located on 8q24.3. However, all three of these showed down-regulation in node-positive as compared with node-negative for any genes located on chromosome regions 8q22-23 and 8q24-qter. Four transcripts were present including three located on 8q24.3. However, all three of these showed down-regulation in node-positive as compared with node-negative for any genes located on chromosome regions 8q22-23 and 8q24-qter.
To determine whether the differentially expressed genes associate with binary class comparison of node-negative and node-positive cases are actually being determined by grade, we did an analysis of grade 1 versus grade 3 cases. In this analysis, we identified genes differentially expressed by at least 2-fold at \( P < 0.005 \). When the list of differentially expressed genes for the grade analysis was compared with the differentially expressed transcripts identified in the supervised analysis of node status, there was no overlap of the two gene lists (see Supplementary Data). This suggests that although cases with node metastasis are associated more often with grade 3 tumors, the differentially expressed genes are unique when compared with the gene expression profile associated with cases having positive nodes.

In conclusion, our results suggest that there are distinct gene expression profiles for endometrial cancer with and without lymph node metastasis. Our supervised analysis has identified genes that are differentially expressed in endometrioid endometrial cancers with lymphatic spread. Although the use of global gene expression analysis of tumors is not likely to have clinical applications because of cost-effectiveness, the use of expression analysis limited to a smaller group of genes that are highly predictive of outcome. Our findings suggest that a larger project aimed at the analysis of preoperative endometrial biopsy or dilation and curettage specimens is warranted.

References


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