

Global Expression Analysis of Cancer/Testis Genes in Uterine Cancers Reveals a High Incidence of *BORIS* Expression

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Abstract Purpose: Cancer/testis (CT) genes predominantly expressed in the testis (germ cells) and generally not in other normal tissues are aberrantly expressed in human cancers. This highly restricted expression provides a unique opportunity to use these CT genes for diagnostics, immunotherapeutic, or other targeted therapies. The purpose of this study was to identify those CT genes with the greatest incidence of expression in uterine cancers.

Experimental Design: We queried the expression of known and putative CT gene transcripts (representing 79 gene loci) using whole genome gene expression arrays. Specifically, the global gene expressions of uterine cancers ($n = 122$) and normal uteri ($n = 10$) were determined using expression data from the Affymetrix HG-U133A and HG-U133B chips. Additionally, we also examined the *brother of the regulator of imprinted sites* (*BORIS*) transcript by reverse transcription-PCR and quantitative PCR because its transcript was not represented on the array.

Results: Global microarray analysis detected many CT genes expressed in various uterine cancers; however, no individual CT gene was expressed in more than 25% of all cancers. The expression of the two most commonly expressed CT genes on the arrays, *MAGEA9* (24 of 122 cancers and 0 of 10 normal tissues) and *Down syndrome critical region 8* (*DSCR8*)/*MMA1* (16 if 122 cancers and 0 of 10 normal tissues), was confirmed by reverse transcription-PCR methods, validating the array screening approach. In contrast to the relatively low incidence of expression of the other CT genes, *BORIS* expression was detected in 73 of 95 (77%) endometrial cancers and 24 of 31 (77%) uterine mixed mesodermal tumors.

Conclusions: These data provide the first extensive survey of multiple CT genes in uterine cancers. Importantly, we detected a high frequency of *BORIS* expression in uterine cancers, suggesting its potential as an immunologic or diagnostic target for these cancers. Given the high incidence of *BORIS* expression and its possible regulatory role, an examination of *BORIS* function in the etiology of these cancers is warranted.

Cancer of the corpus uteri is the most frequent gynecologic malignancy in the United States. The American Cancer Society estimates that 41,200 of these cancers will develop in 2006, and ~7,350 of these women will die (1). It is therefore important

to develop novel strategies for early detection, diagnosis, and treatments for these cancers. Of particular interest, immunotherapy that targets antigens specifically expressed in cancer cells but not in normal tissues is one such possibility. Cancer/testis (CT) genes offer an appealing class of targets. In normal tissues, CT genes are predominantly expressed in the germ cells of the testis, placenta, or ovary but are often aberrantly expressed in various cancers. CT genes have recently been expertly reviewed (2–4). Some of these gene products are immunogenic in cancer patients, designated CT antigens, and are promising vaccine targets in cancer therapy (2). Additionally, these genes encode products that are possible targets for small molecule-based drugs.

Only limited studies on the expression incidence of various known CT genes in uterine cancers are reported. Resnick et al. examined the expression of *MAGEA4* and *NY-ESO-1* in a variety of uterine cancers and observed that a high proportion of uterine carcinosarcomas (91%) and papillary serous cancers (63%) expressed these antigens (5). In contrast Chitale et al. evaluated *NY-ESO-1* by immunohistochemistry and found that only 6% of endometrial carcinomas expressed the gene (6). Various other CT genes have been evaluated in endometrial cancers, including

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MAGEA4, *MAGEA3*, *MAGE-C1* (*CT7*), *SSX*, and *CAGE* (6–8). To more comprehensively examine this topic, we did a screen for CT genes using the gene expression data obtained from microarray analysis of 122 uterine cancers and also from 10 normal uteri. A similar screen identifying germ line genes was recently reported (9). In addition, we used individually tailored reverse transcription-PCR (RT-PCR)/real-time quantitative RT-PCR assays to confirm findings from the array data as well as to examine CT genes not present on the array chips. The goal of these screens was to identify those CT genes with the highest incidence of expression in uterine cancers.

Materials and Methods

Clinical specimens. Flash frozen uterine cancers ($n = 122$) were obtained from patients undergoing hysterectomy at the Duke University Medical Center. None of the patients had received preoperative chemotherapy or radiation. In addition, samples of histologically confirmed normal endometrium ($n = 10$) 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 exempted by the National Cancer Institute Institutional Review Board. Tissue samples were subjected to RNA isolation using TRIzol (Invitrogen, Carlsbad, CA) and an additional purification using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's recommendation. Following isolation of RNA, the integrity of each RNA sample was verified by denaturing gel electrophoresis.

Gene expression array. We studied gene expressions using Affymetrix Human Genome HG-U133A and HG-U133B Gene Chip expression arrays (45,000 array features covering >28,000 UniGene clusters). Five micrograms of total RNA from each sample were labeled using the bioarray high-yield transcript kit according to manufacturer's conditions (ENZO, Farmingdale, NY). Labeled RNAs were hybridized and washed according to manufacturer's recommendations (Affymetrix, Inc., Santa Clara, CA). Initial gene expression data analysis was done using Microarray Suite 5.0 software (Affymetrix 2001). All arrays were normalized to a trimmed mean transcript signal level of 500 counts (absent and present call procedure). Expression values for each sample for each element of the filter are contained in the Supplementary Material.

Statistical calculations. All statistical calculations were done on the logarithmic values of signals or ratios. The expression ratios of *MAGEA9* and *DSCR8* to β -actin are shown for mixed mesodermal tumor, papillary serous cancer, and endometrioid cancer. Comparison of the geometric mean expression values of Taqman data indicates that mixed mesodermal tumors are 8-fold higher than endometrioid cancers, and that papillary serous cancers are 19-fold higher than endometrioid cancers. The statistical significance of these differences was tested using two-tailed Student's *t* tests. *MAGEA9* expression is found to be significantly different in papillary serous cancers compared with endometrioid cancers at $P < 0.03$ (papillary serous/endometrioid = 16.7), whereas all other comparisons indicated $P_s > 0.05$ (*MAGEA9*: mixed mesodermal tumor/endometrioid = 8.5, $P = 0.127$; *DSCR8*: mixed mesodermal tumor/endometrioid = 19.0, $P = 0.056$; papillary serous/endometrioid = 11.7, $P = 0.1$). Similarly, *BORIS* expression is higher in mixed mesodermal tumor and papillary serous cancers compared with endometrioid cancers with the geometric mean ratios of mixed mesodermal tumor/endometrioid = 132 and papillary serous/endometrioid = 18, respectively. Both mixed mesodermal tumor and papillary serous cancers are found to be significantly different from endometrioid cancers, with P values of 2.8×10^{-8} and 0.0018, respectively, whereas mixed mesodermal tumor is not significantly different from papillary serous cancers, although the mixed mesodermal tumor/papillary serous ratio is 7.1. Examination of endometrioid cancers indicated progressive increase in *BORIS* expression with grade,

although the differences among grades 1, 2, and 3 are not statistically significant ($P \geq 0.1$).

CT antigen filter. We created a list of potential CT genes based on an examination of the literature and gene databases. Although many genes described in the literature have been described as CT genes, few have had a thorough examination of their expression in a wide variety of fetal and adult tissues. In addition, many have not been described in the literature as being expressed aberrantly in cancer but rather show strong sequence homology to other known CT genes. We therefore queried the data from the gene arrays for both expression in cancers and also in 10 normal uteri. Potential CT genes were excluded when expression was detected in one or more normal uteri. Genes contained in this filter are contained in Supplementary Table S1.

Real-time PCR and RT-PCR. Total RNA from each sample was used to make first-strand cDNA using a commercial kit (Invitrogen). The quality of all cDNAs was assured by confirmed expression of the housekeeping genes *β -actin* (*ACTB*) and *GAPDH*. β -Actin and *GAPDH* assays were obtained from ABI (Foster City, CA). First-strand cDNA was used to assess *BORIS* expression using quantitative real-time PCR. The relative concentration of *BORIS* was determined using *ACTB* as a reference, as described by Vatolin et al. (10). *BORIS* real-time PCR primers and probe with corresponding position in the reference sequence (NM_080618) were forward primer, CCCATGTGCCAC-CATCA (1374-1391) and reverse primer, AGCATGCAAGTTGCGCATAT (1438-1418). Taqman MGB probe, FAM-ACGGAAAAGCGACCTAC (1396-1412). *BORIS* real-time results were confirmed by conventional RT-PCR using primers described in a subset of samples (11). Similarly, relative levels of *MAGEA9* and *DSCR8* were determined using commercially available probe and primer sets (Applied Biosystems, Foster City, CA). Primers used for detection of *NY-ESO-1* and *LAGE* have been described (12). Normal human testis RNA (Clontech, Palo Alto, CA) served as positive control for all reverse transcription-PCR. Samples from normal human tissues were obtained from Clontech. In addition to the 10 normal uterine biopsy RNAs, commercially available RNA from uterus was also used as negative control (Clontech).

Results

Creation of a CT gene filter. We examined the literature and gene databases to create a list of transcripts that were previously described as CT genes or that had strong sequence similarity to known CT gene family members but had not yet been described as a CT gene. CT genes have recently been extensively reviewed, and the CT gene database⁵ contains over 80 CT genes (2–4, 13). However, some are not confirmed as aberrantly expressed in cancer (e.g., *SPANX-N* family), and candidate novel CT genes likely remain to be identified (13–15). We therefore created a list based on reported literature as well as upon sequence homology. This potential CT gene list was compared with the known transcripts represented on the Affymetrix HG-U133A and HG-U133B chips. A total of 79 loci of potential CT genes were represented by at least one probe set on the gene chips. These transcripts and corresponding Affymetrix probes are listed in Supplementary Table S1. Several interesting CT genes and potential CT genes by homology were not represented on these particular array chips, including *BORIS*, *XAGE4*, *CAGE*, and the recently described *SPANX-N* (new members N1-N5) and *CT45* families (five of six genes; refs. 13–15).

Determination of CT genes expressed in uterine cancers by gene expression arrays. Our group has previously completed gene expression analysis of 122 uterine cancers and 10 normal uteri using the Affymetrix HG-U133A and HG-U133B gene chips

⁵ <http://www.cancerimmunity.org/CTdatabase/#Ctlist>

Endometrioid

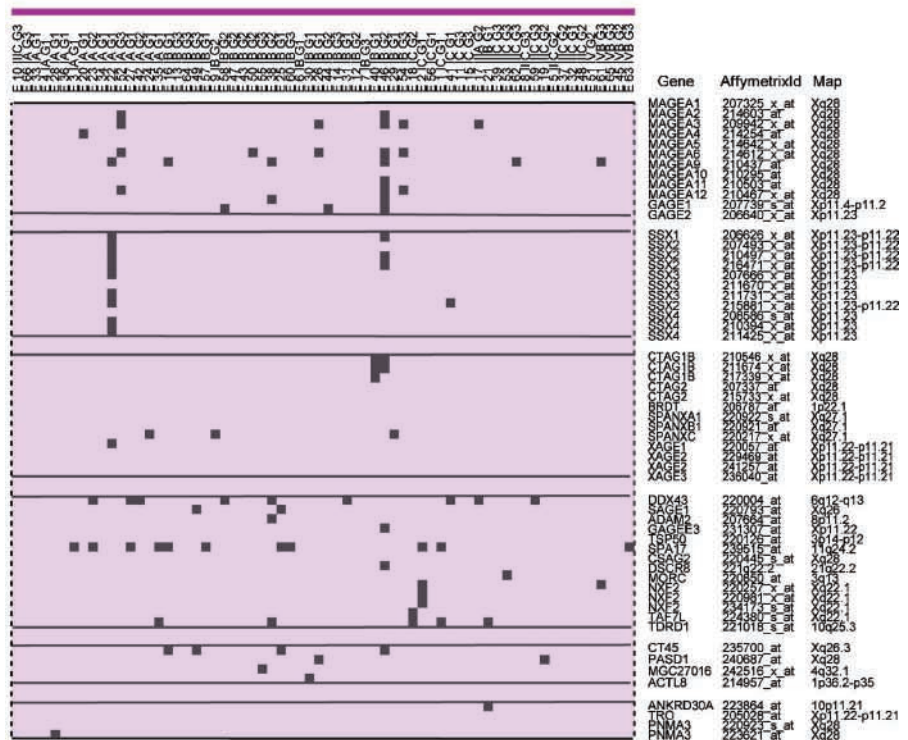
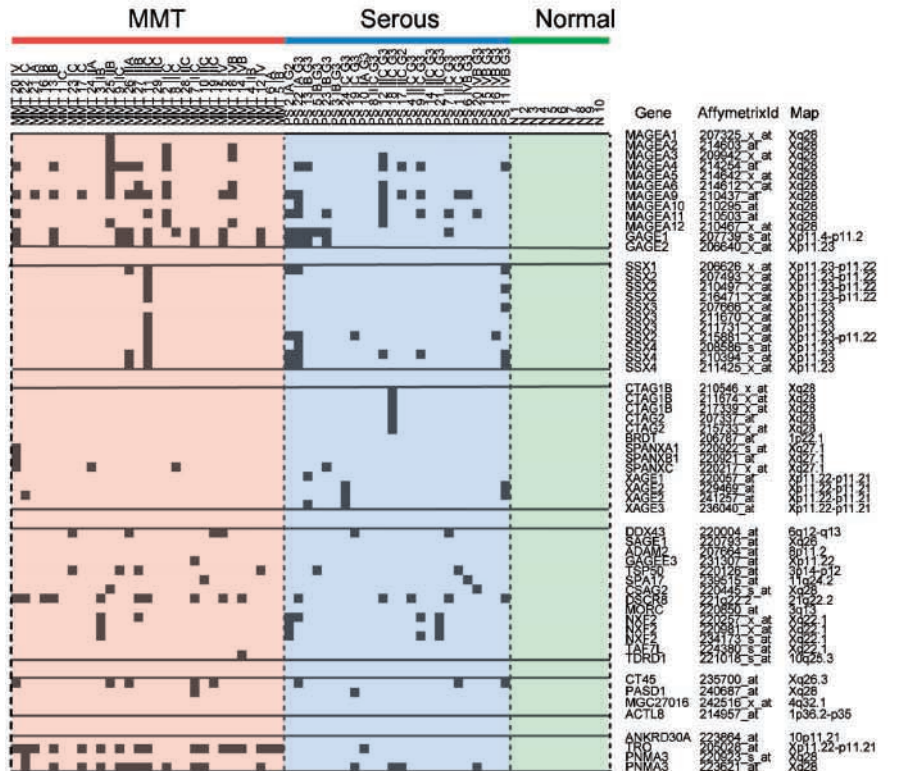


Fig. 1. Graphical representation of CT genes expressed in individual endometrioid tumors. Black boxes, expression as defined in the text for various samples. Clear cell (CC), mucinous (M), endometrioid (E), mixed mesodermal tumors (MMT), papillary serous (PS), and normal (N) endometrial histology were grouped together with stage and grade (when known) of each samples listed. CT genes absent on all arrays: *MAGEB1*, *MAGEC2*, *MAGEC3*, *SSX5*, *PAGE-5*, *ACRBP*, *LUZP4*, *TFDP3*, *JARID1B*, *LDHC*, *DKKL1*, *SPO11*, *FTHL17*, *TAF7L*, *TDRD6*, *TPTE*, *THEG*, *LOC348120*, *LOC196993*, *FLJ25339*, *MGC27016*, *ADAM29*, *NALP4*, *SSTK*, *CTAGE5*, *CXorf48*, and *KM-HN-1*.



CT genes absent on all arrays:
MAGEB1 *MAGEC2* *MAGEC3* *SSX5* *PAGE-5* *ACRBP* *LUZP4* *TFDP3* *JARID1B*
LDHC *DKKL1* *SPO11* *FTHL17* *TAF7L* *TDRD6* *TPTE* *THEG* *LOC348120* *LOC196993*
FLJ25339 *MGC27016* *ADAM29* *NALP4* *SSTK* *CTAGE5* *CXorf48* *KM-HN-1*

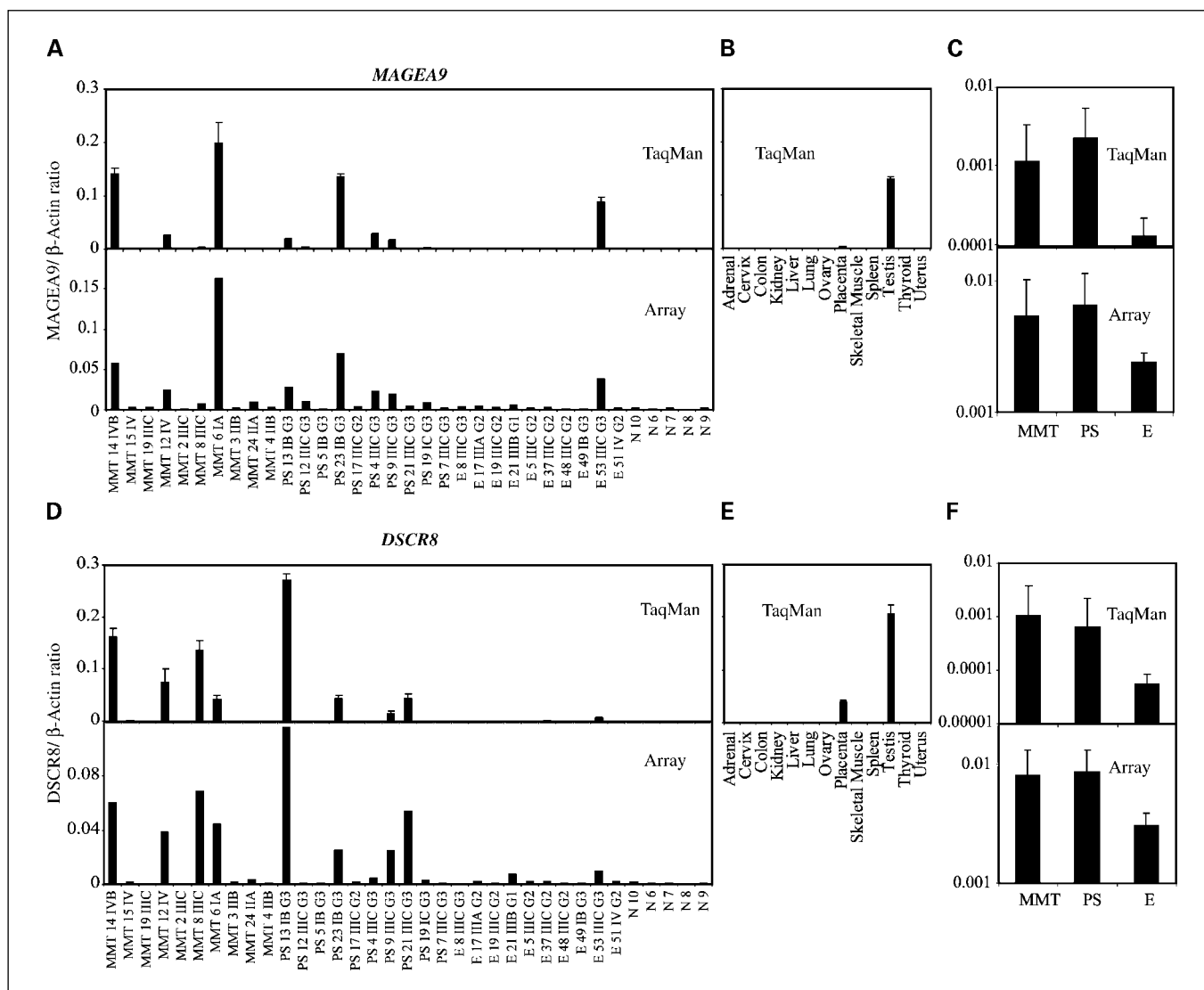


Fig. 2. Gene expression data for MAGEA9 and DSCR8 CT genes in uterine and normal tissue samples. Concordance of microarray based probe data and real-time PCR derived data show strong concordance of MAGEA9 (A) and DSCR8 (D). Real-time PCR (Taqman) data for MAGEA9 (B) and DSCR8 (E) in a panel of 13 normal human tissues showing robust expression in testis, limited expression in placenta, and no detectable expression in other tissues examined. Expression of MAGEA9 (C) and DSCR8 (F) for each histologic type: E, endometrioid; MMT, mixed mesodermal tumors; PS, papillary serous.

(16, 17). These samples included 63 endometrioid endometrial cancers, 24 papillary serous cancers, 29 mixed mesodermal tumor of the uterus, 2 clear cell cancers, and 1 mucinous cancer. These expression data were analyzed using the potential CT gene list to identify those transcripts present in some subset of tumors but not in normal uteri. We applied stringent criteria in our selection of genes by using the Affymetrix absent/present call in making this determination. Although this analysis feature has the potential to exclude some genes that are truly expressed at low levels, it provides strong evidence for true gene expression. Initially, we examined those transcripts that were expressed in the normal uteri samples and excluded any that were given present calls. We used the most stringent cutoff and excluded the gene even if it was found expressed in only one of the 10 normal uteri. These data are depicted in Fig. 1, and data are contained in Supplementary Table S1. The genes involving the greatest numbers of cancers as detected by this screen were

MAGEA9 (24 of 122), PNMA3 (17 of 122), and DSCR8 (also called MMA1; 16 of 122).

Expression of MAGEA9 and DSCR8 in uterine cancers and normal human issues are consistent with other CT genes. We next examined several transcripts by reverse transcription-PCR to confirm the array findings. Aberrant expression of MAGEA9 was not previously reported; yet, this gene is considered a CT gene based on strong homology to other MAGEA family members (18). We therefore validated its expression by alternative methods. An analysis of 30 cancers and 5 normal endometria by reverse transcription-PCR (data not shown) and quantitative real-time PCR confirmed expression of MAGEA9 in a subset of uterine cancers (Fig. 2A). We also examined MAGEA9 expression in a panel of 13 normal human tissues and confirmed expression only in testis and placenta (Fig. 2B), establishing MAGEA9 as a CT gene. Like many of the CT genes found expressed in uterine cancers, activation of expression of

these gene was more prominently found in tumors of serous or mixed mesodermal tumor histologies (Fig. 2C).

Most CT genes are located within specific clusters located on the X chromosome (19). An interesting observation from the array screen was the number of tentatively defined CT genes that were excluded due to expression in normal uterus included almost all of those located on the autosomes. However, several genes located on the autosomes were not excluded, including *DDX43* (6q12-q13) and *DSCR8* (21.q22.2). Of these, *DSCR8*, one of several alternatively spliced members of the *MMA1* family of CT genes (20), was expressed in the greatest number of uterine cancers; thus, we chose to validate its expression as well. RT-PCR (data not shown) and real-time PCR analysis confirmed expression of *DSCR8* in a subset of cancers and lack of expression in normal uterus (Fig. 2D). *DSCR8* also possessed a testis and placental restricted expression pattern in a screen of 13 normal tissues (Fig. 2E). Similar to *MAGEA9* and many of the other CT genes found expressed on the array, *DSCR8* was more frequently expressed in serous and mixed mesodermal tumor histologies (Fig. 2F). In addition, to these two transcripts, we also evaluated expression of the *SPANXC* gene that showed a relatively infrequent incidence of expression in the cancer set and confirmed this data by RT-PCR (data not shown).

In summary, RT-PCR correctly identified frequently expressed CT genes (i.e., *MAGEA9* and *DSCR8*) as well as a CT gene chosen that was infrequently expressed (i.e., *SPANXC*).

BORIS transcript expression in normal and malignant uterine tissues. We examined the newly described *BORIS* CT gene for which probes were not present on the array chips. The recently described *BORIS* was found expressed in a variety of cancer cell types (*in vitro* cell lines and primary tumors) and may play a role in the etiology of these cancers (11, 21). We examined *BORIS* expression using both conventional RT-PCR as well as quantitative real-time PCR in the same set of cancers that were examined by microarray as well as four additional tumors. Interestingly, we found a very high incidence of expression of *BORIS* in these cancers (Fig. 3A). Specifically, 73 of 95 endometrial cancers and 27 of 31 mixed mesodermal tumors expressed *BORIS* transcript. Importantly, we found no expression of *BORIS* in normal endometrial samples, or in 20 benign uterine leiomyomas and their matched normal myometrium (Supplementary Fig. S1).

Interestingly, *BORIS* expression was detected in many endometrioid endometrial cancers (Fig. 3A and C); however, expression levels seemed to be lower in many cases. We examined the relative level of transcript detected in these

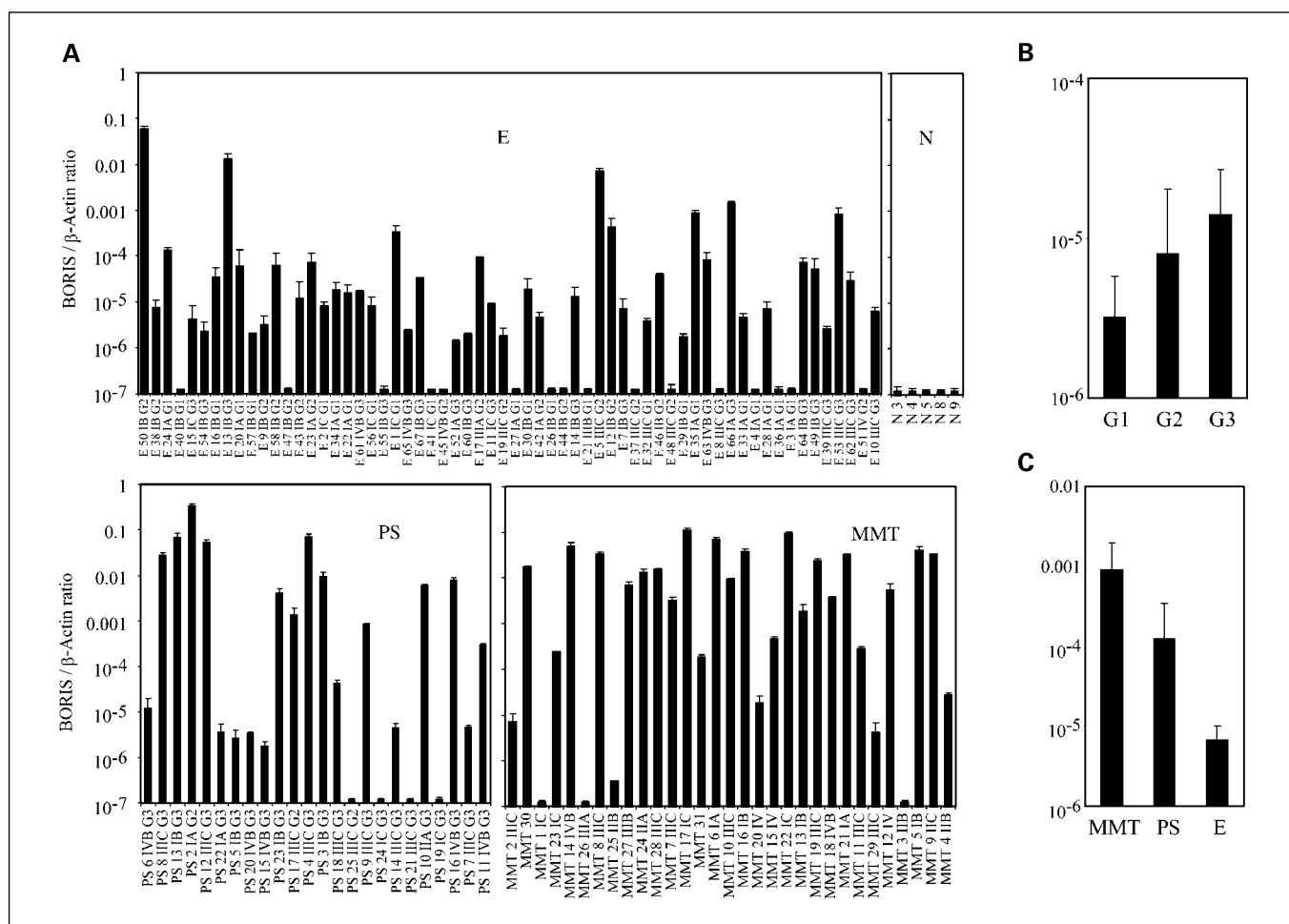


Fig. 3. *BORIS* expression in uterine samples. Real-time PCR expression of *BORIS* transcript plotted as ratio with *ACTB*, showing frequent *BORIS* expression in malignant endometrial samples (E, endometrioid; MMT, mixed mesodermal tumors; PS, papillary serous) and absent expression in normal endometrial samples (A). *BORIS* transcript levels in endometrioid endometrial cancers by tumor grade (B). *BORIS* transcript levels for each histology (C).

histologic types by comparing the geometric mean of their expression and found that both papillary serous cancers and mixed mesodermal tumors were significantly different from endometrioid cancers. Specifically, the geometric mean ratio of mixed mesodermal tumor/endometrioid is 132 and of papillary serous/endometrioid is 18. Both mixed mesodermal tumor and papillary serous cancers are found to be significantly different from endometrioid, with P values of 2.8×10^{-8} and 0.0018, respectively. To further examine this issue, we examined the influence grade might have on *BORIS* expression in these cancers and found that on average, *BORIS* was more highly expressed in tumors with high grade (Fig. 3B), but this difference was not significant ($P = 0.098$).

Discussion

In this report, we describe a screen of multiple CT genes in a large panel of uterine cancers. We used defined criteria to sort our microarray data imposing strict cutoffs in numbers of normal endometria permitted to express a given transcript. Even within these defined limits, many CT genes were shown to be expressed in endometrial cancers; however, most of these occurred in the minority of samples. Several potential CT genes contained more than one probe set on the chips, and these chips sometimes provided differing data. For example, two probe sets were present for *MAGE-D4*, one of which was excluded due to expression in all normal endometria, whereas the other was not. The remaining probe set showed a high frequency of expression in mixed mesodermal tumors, infrequent expression in serous and endometrioid cancers, and no expression in normal uterine samples (Fig. 1; Supplementary Table S1). These data warrant further investigation of this *MAGE* family member in uterine mixed mesodermal tumors. Similarly, three of four probe sets for the *Trophinin* (*TRO*) gene were excluded due to expression in normal endometria. *TRO* is well known to be expressed in endometrium but was included in the screen because it shares homology to *MAGE* family members. The locus has multiple transcripts that can encode *MAGE*-like peptides with functions during gametogenesis (22). The data for the single probe set not excluded due to normal endometrial expression are contained in Fig. 1. This particular *TRO* isoform was widely expressed in the majority (17 of 29, 58%) of uterine mixed mesodermal tumors, suggesting its importance in this aggressive histologic subtype of uterine cancer.

Somewhat surprising were the results for *NY-ESO-1* (*CTAG1*) and *MAGEA4*. Only two cancers exhibited *NY-ESO-1* expression, and only 14 cancers expressed *MAGEA4* based on our microarray data. This finding is in contrast to results obtained by Resnick et al. who observed higher incidence of expression using immunohistochemistry (5). However, our data are in concordance with another report that found only 6% of endometrial cancers expressed *NY-ESO-1*, and 23% expressed *MAGEA4* (6). We chose to re-evaluate *NY-ESO-1* expression in our cancers by conventional non-quantitative reverse transcription-PCR. These data tracked our microarray findings, as we confirmed expression in these 2 cancers but not 20 others that were negative by array (data not shown). *MAGEA* family members share very high amino acid homology yet contain considerable variation at the nucleotide level. The differences in this previous report may

be due to differences in methodology (immunohistochemistry versus oligonucleotide array) or in specificity. Our data were obtained from oligonucleotide array that has the ability to distinguish between family members. Alternatively, low levels of transcript undetectable by array technologies may not be reflective of highly stable and abundant protein detected by immunohistochemistry.

Several interesting genes with a high incidence of expression in cancers were excluded from our analysis due to low levels of expression in several normal samples. Among these was the *PRAME* antigen, excluded due to low but reliably detected expression in normal uterine samples. *PRAME* was highly expressed in many of the endometrial cancers, and unlike the other CT genes that tended to be expressed in more aggressive serous and mixed mesodermal tumor histologic types, *PRAME* showed a high incidence and level of expression in many early-stage endometrioid cancers as well as serous and mixed mesodermal tumor types. Furthermore, *PRAME* was identified as a cancer biomarker based on its increased level of expression in a comparison of normal endometrium and endometrial cancer.⁶ This expression in the most common histologic type of endometrial cancer deserves further investigation, including analysis of the relative levels of transcript and protein in normal and malignant uterine tissue. Recent progress in the understanding of the role of *PRAME* in modulating tumor progression through retinoic acid signaling further highlights the need for more study in this area (23).

Importantly, we identify for the first time a high incidence of expression of the *BORIS* gene. *BORIS* was expressed in the majority of uterine cancers examined and was not found expressed in normal uterine samples. These expression data are consistent with other evidence in the literature that *BORIS* is a true CT gene in which expression occurs only in testis and malignancies and not other tissues (11). The fact that *BORIS* expression was frequently detected in cancers and not in normal adult female tissues raises the possibility that *BORIS* represents a novel target for immunotherapy of endometrial cancers. Additionally, *BORIS* expression could be used as a potential screening marker for presence of malignant uterine disease or for monitoring treatment response in patients with endometrial cancer.

Although the normal function of most CT genes is unknown, *BORIS* contains an almost identical 11 zinc-finger region present in the CTCF transcription factor. Based on this homology, *BORIS* would be predicted to bind similar DNA elements as CTCF (11, 21). CTCF functions in the cell are numerous, including regulation of many epigenetically regulated loci, such that promoter competitions might disrupt the normal expression of many genes (24–27).

Indeed, recent data indicate that *BORIS* can modulate the expression of several CT genes in normal human dermal fibroblasts as well as lung cancer cells (10, 28). Specifically, Vatolin et al. found that ectopic *BORIS* expression could repress a variety of CT genes in neonatal dermal fibroblasts (10). Similarly, Hong et al. described the ability of *BORIS* to repress the *NY-ESO-1* CT gene in lung cancer cells (28). Functionally, *BORIS* was found to replace the CTCF transcription factor on CT gene promoters normally necessary for

⁶ G.V.R. Chandramouli and J.I. Risinger, unpublished data.

maintaining these loci in a silenced state. Although some of these data were obtained from *in vitro* cell culture models, it is possible that BORIS can aberrantly modulate the expression of a variety of genes (not just CT genes) normally regulated by CTCF in cancers and influence the carcinogenic process. Further examination of this concept is warranted.

In addition, to the BORIS gene, our data identified a variety of CT genes that are expressed in uterine cancers. Of these, we identify for the first time expression of MAGEA9 in any cancer

tissue. MAGEA9 was originally identified based on homology to other MAGEA family members, but to date, no evidence existed as to its expression in cancers. Of the CT genes other than BORIS, MAGEA9 was expressed in the most uterine cancers with highest incidence in the mixed mesodermal tumors (11 of 29, 38%) and papillary serous cancers (7 of 24, 29%). This report verifies this MAGE family member as a true CT gene frequently expressed in these histologic types of uterine cancer with unfavorable prognosis.

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