Molecular characterization of human adenomyosis

A.Hever1,3, R.B.Roth1, P.A.Hevezi1, J.Lee1, D.Willhite1, E.C.White1, E.M.Marin2, R.Herrera2, H.M.Acosta2, A.J.Acosta2 and A.Zlotnik1

1Neurocrine Biosciences, Discovery Biology, San Diego, CA, USA and 2School of Medicine, University of Baja California, Mexicali, Mexico

To whom correspondence should be addressed at: Neurocrine Biosciences, Discovery Biology, 12790 El Camino Real, San Diego, CA 92130, USA. E-mail: ahever@neurocrine.com

Adenomyosis is a common gynaecological disorder characterized by the abnormal growth of endometrium into the myometrium and myometrial hypertrophy/hyperplasia. Uterine fibroids are benign neoplasms of the myometrium, and they represent a diagnostictic pitfall for adenomyosis. In this study, we have used the genome-wide Affymetrix U133 Plus 2.0 microarray platform to compare the gene expression patterns of adenomyosis, uterine fibroids, normal endometrium and myometrium. Unsupervised principal component analysis (PCA) revealed that these four tissue types could be segregated from one another solely based on their gene expression profiles. Analysis of variance (ANOVA), followed by Tukey means separation test, significance analysis of microarrays (SAM) and 2-fold change threshold, identified 7415 probe sets as differentially expressed among the four groups of samples. Supervised cluster analysis based on these probe sets clustered adenomyosis most closely with endometrium and uterine fibroids with myometrium, consistent with the anatomic origin of these two diseases. The Tukey means separation post hoc testing found 2073 probe sets altered between adenomyosis and normal endometrium or myometrium, and 2327 probe sets altered in expression when comparing uterine fibroids with myometrium. Using Ingenuity Pathways Analysis (IPA), we found 9 highly significant functional networks in adenomyosis and 10 in uterine fibroids. Notably, the top network in both cases was associated with functions implicated in cancer and cell death. Finally, we compared the gene expression profiles of adenomyosis and uterine fibroids and identified 471 differentially expressed probe sets that may represent potential biomarkers for the differential diagnosis of these diseases.

Key words: adenomyosis/Affymetrix/Ingenuity/uterine fibroids

Introduction

Adenomyosis is a common benign disease of the uterus that can arise as diffuse and/or focal, tumor-like growth (also called adenomyoma) (Ferenczy, 1998). Despite its frequent occurrence, the precise aetiology of adenomyosis is still unknown. The conventional view is that it results from the abnormal growth and invasion of endometrium into the myometrium (Ferenczy, 1998). Microscopically, adenomyosis exhibits ectopic endometrial glands and stroma surrounded by hypertrophic and hyperplastic myometrium. Regarding the role of myometrium in the development of adenomyosis, two theories have emerged: (i) myometrial smooth-muscle hypertrophy and hyperplasia is a response of the myometrium to the (abnormal) presence of endometrial cells and (ii) myometrial smooth-muscle dysfunction develops not as a consequence but rather as a primary defect in adenomyosis (Ferenczy, 1998; Parrott et al., 2001).

Adenomyosis preferentially affects multiparous women in their reproductive and perimenopausal years, and it has also been reported in post-menopausal breast cancer patients treated with tamoxifen (Cohen et al., 1997). Approximately, two-thirds of adenomyosis patients show symptoms, such as dysmenorrhea, menorrhagia and anaemia, and they have a high incidence of early-pregnancy-stage miscarriages (Olive et al., 1982). In addition, some reports have suggested a possible relationship between adenomyosis and endometrial adenocarcinoma (Rubet al., 2004).

The pre-operative diagnosis of adenomyosis is only suggestive at best and most often is either not made or overdiagnosed (Ferenczy, 1998). The most frequent problem is the differentiation of adenomyosis from uterine fibroids (also called leiomyoma). Although these two diseases have similar clinical symptoms, their surgical treatment differs. Uterine fibroids usually compress the surrounding myometrium, thus creating a pseudocapsule from which the tumor can easily be enucleated at surgery (Ferenczy, 1998; Tamai et al., 2005). In contrast, adenomyosis interdigitates with normal smooth muscle, making surgical excision difficult or impossible, and therefore, the definitive treatment for this type of lesion is usually hysterectomy (Ferenczy, 1998; Tamai et al., 2005). A correct pre-operative diagnosis may be important in those patients who have planned for preservation of fertility. Several non-invasive imaging techniques have proven useful in diagnosing adenomyosis (Reinhold et al., 1998). Magnetic resonance imaging (MRI) is currently the most accurate method for distinguishing adenomyosis from leiomyoma. In MRI, adenomyosis appears with a poorly defined border, minimal mass effect, elliptical rather than round configuration and absence of dilated vessels at the margin of the lesion, and these characteristics differentiate it from leiomyoma (Reinhold et al., 1999; Tamai et al., 2005). However, focal adenomyosis may be indistinguishable from leiomyoma even with MRI (Togashi et al., 1989; Reinhold et al., 1999; Ascher et al., 2003; Tamai et al., 2005). In addition, MRI is an expensive imaging technique, limiting access to affluent health care systems (Wood, 1998).

In this study, we sought to better understand the pathobiology of adenomyosis at the molecular level and to identify potential diagnostic markers that could represent new options for the differential diagnosis.
of adenomyosis from uterine fibroids. We approached the problem by investigating expression profiles using DNA microarray technology. Microarray analysis is a powerful tool that has been used to analyze several cancers and other diseases, including benign gynecological conditions, such as uterine fibroids (Tsibris et al., 2002; Chegini et al., 2003; Skubitz and Skubitz, 2003; Wang et al., 2003; Catherino et al., 2004; Quade et al., 2004; Arslan et al., 2005; Luo et al., 2005) and endometriosis (Eyster et al., 2002; Arimoto et al., 2003; Kao et al., 2003; Matsuzaki et al., 2004). There are, however, no reports describing microarray analysis of human adenomyosis. To this end, we compared the gene expression profiles of adenomyosis, uterine fibroids, normal endometrium and myometrium, using Affymetrix U133 Plus 2.0 GeneChips that provide genome-wide coverage.

Materials and methods

**Tissue specimens**

Ten adenomyosis (A2 and A10, follicular phase; A1, A4, A6, A7, A8 and A9, luteal phase; A3 and A5, unknown phase) and six uterine fibroid (UF1–UF3, follicular phase; UF4–UF6, luteal phase) samples were obtained from Zion Diagnostics (Hawthorne, NY, USA). All donors were Caucasian and were taking no medications, and none received hormone therapy before surgery. Adenomyosis donors A3, A5, A6, A7, A8 and A10 also had co-existing uterine fibroids. Following surgical removal of the uterus (no later than 30 min), tissue samples were snap-frozen in liquid N2 and were stored at −80°C. Five control myometrium (myo1 and myo2, follicular phase; myo3–myo5, luteal phase) samples were obtained from females who underwent surgery for uterine fibroids. Haematoxylin and eosin slides of all tissue samples were evaluated by the project pathologist who confirmed the diagnosis of disease samples and the absence of pathology of the control samples. Gene expression data of six control endometrium samples (M182 = endo 1, M165 = endo 2 and M169 = endo 3, follicular phase; M153 = endo 4, G98A = endo 5 and M163 = endo 6, luteal phase) were downloaded from the publicly available NCBI GEO gene expression database [Accession ID: GSE4888 (Talbi et al., 2006)].

**Microarray gene expression profiling**

Total RNA was isolated using a standard Trizol protocol, checked for integrity by gel electrophoresis and further purified using RNeasy columns (Qiagen, Valencia, CA, USA). Five micrograms of total RNA from each sample was used to direct first-strand cDNA synthesis using a T7-oligo(dT)24 primer and PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA). Following second-strand synthesis and clean-up with a Qiaquick spin column (Qiagen), the double-stranded cDNA was used in a MEGAscript T7 RNA polymerase in vitro transcription (IVT) reaction (Ambion, Austin, TX, USA), containing biotin-labelled ribonucleotides, CTP and UTP. The resulting labelled cRNAs were hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) overnight, washed and scanned according to the manufacturer’s protocol using a GeneChip Scanner 3000 and GeneChip Operating Software (GCOS; Affymetrix).

**Data processing and statistical analysis**

Affymetrix cel files were uploaded to Genedata Expressionist Pro3.0 (Basel, Switzerland) and subsequently background corrected, normalized and polished using robust multi-chip average (RMA). Unsupervised principal component analysis (PCA) (Raychaudhuri et al., 2000) was performed using all probe sets with a correlation matrix following z-normalization. RMA-processed data (Supplementary Table I) was subsequently uploaded to GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA, USA), and a one-way analysis of variance (ANOVA) was performed followed by Tukey means separation post hoc testing. To estimate the number of genes found by chance, we performed significance analysis of microarrays (SAM; Stanford University) (Tusher et al., 2001) using RMA data with the delta set at 0.39 and a false discovery rate (FDR) of 0.01%. Finally, a fold-change filter was applied where at least one of the following pair-wise comparisons had to show a 2.0-fold or greater difference in expression: adenomyosis versus endometrium, adenomyosis versus myometrium, uterine fibroids versus myometrium, adenomyosis versus uterine fibroids or myometrium versus endometrium. This resulted in a filtered set of 7415 probe sets (Supplementary Table II) meeting our criteria for significance. Supervised two-dimensional (2D) hierarchical clustering was performed with GeneSpring GX 7.3 using the Pearson correlation metric and average linkage following per gene normalization.

**Quantitative (real-time) polymerase chain reaction (Q-PCR)**

Total RNAs from diseased or normal samples were converted into single-stranded cDNAs using the High-Capacity cDNA Archive Kit (Applied Biosystems Group, Foster City, CA, USA), according to the manufacturer’s instructions. PCR was performed on the ABI PRISM 7900HT Sequence Detection System in 384-well plates. TaqMan Universal PCR MasterMix and Assays-on-Demand Gene Expression probes (Applied Biosystems) were used for the PCR step, according to the manufacturer’s instructions. Expression values obtained were corrected for loading by measuring 18S RNA relative expression levels and quantified by converting the cycle threshold (Ct) value into a numerical value by using the following formula: expression value = 2^(ΔΔCt) (Heguera et al., 2003).

**Ingenuity Pathways Analysis**

The probe sets differentially expressed in adenomyosis versus normal endometrium or myometrium and in uterine fibroids versus myometrium were used for network analysis. Data sets containing the Affymetrix probe set identifiers and fold changes were uploaded into Ingenuity’s [Ingenuity pathway analysis (IPA)] software (http://www.ingenuity.com). The IPA program searches the Ingenuity Pathway Knowledge Base (IPKB) for interactions (known from the literature) between the uploaded genes and all other genes contained in IPKB and generates a series of networks. The Fisher’s exact test is used to assign statistical significance, and each network’s score is displayed as the −log (P-value). The score represents the probability of finding ‘x’ focus genes or more in a set of ‘n’ genes randomly selected from Ingenuity’s global molecular network. For example, a score greater than 2 indicates that there is a less than 1 in 100 chance (P < 0.01) that genes are assembled into a network because of random chance. In order for a gene to become a network focus gene, it must meet two criteria: (i) the gene must meet all of the criteria specified in the statistical analysis of the gene expression data (e.g. in our study: ANOVA/Tukey/SAM/2-fold filter) and (ii) there must be at least one other full-length, wild-type gene or protein in the IPKB that interacts (directly or indirectly) with this gene. Networks including 35 focus genes are highly significant (score ≥ 35). Biological functions were also assigned to each network.

**Results**

**Comparison of gene expression profiles by PCA (unsupervised)**

We applied unsupervised PCA to the RMA data (Supplementary Table I) to establish the interrelationships among the tissue samples used in our study. PCA is a statistical method that can be used to reduce complex data sets with multiple variables into significantly smaller numbers of variables (known as components), which retain the relevant variance information used to distinguish the sample groups from one another. By visualizing projections of these components in low-dimensional spaces, we were able to observe the grouping of samples reflecting underlying patterns in their gene expression profiles. The first three components, which accounted for ~50% variance in our gene expression data, separated adenomyosis, uterine fibroids, endometrium and myometrium samples into four distinct groups (Figure 1).

**Comparison of gene expression profiles by ANOVA/Tukey/SAM (supervised)**

ANOVA, followed by Tukey means separation post hoc testing was performed to identify those probe sets differentially expressed among adenomyosis, uterine fibroids, endometrium and myometrium. To
control the FDR, SAM was applied ($\Delta = 0.39$, FDR $= 0.01\%$). Using this approach, 19896 probe sets were found to be differentially expressed among the four groups analysed. Subsequently, a fold-change filter was set to 2.0, which reduced the number of significant probe sets to 7415 (Supplementary Table II). After SAM and fold-change filtering, the Tukey means separation post hoc testing identified 2941 probe sets as differentially expressed between adenomyosis and endometrium, 2826 probe sets between adenomyosis and myometrium, 2327 probe sets between uterine fibroids and myometrium, 471 probe sets between adenomyosis and uterine fibroids and 4699 probe sets between endometrium and myometrium. The set of 7415 differentially regulated probe sets was used for supervised hierarchical clustering, and the result is shown in Figure 2. The tissue sample dendrogram initially divides adenomyosis and endometrium samples as one group and uterine fibroid and myometrium samples as another group. At the next branch point, all adenomyosis samples segregate from all endometrium samples, and all uterine fibroid samples segregate from all myometrium samples. There are two distinct subgroups within the endometrium and adenomyosis clusters.

**Dysregulated genes and functional networks in adenomyosis and uterine fibroids**

Of the 7415 probe sets identified by our statistical analyses described above, there were 2941 probe sets differentially expressed in adenomyosis versus endometrium and 2826 probe sets in adenomyosis versus myometrium (Supplementary Table II). The number of up- or down-regulated probe sets was similar in adenomyosis versus endometrium: 1633 were up-regulated and 1308 were down-regulated, whereas there were more down-regulated than up-regulated genes in adenomyosis versus myometrium (2241 versus 585 probe sets). As the adenomyosis samples in our study consisted not only of endometrial tissue but also of myometrial tissue, a direct comparison of the gene expression profiles of adenomyosis versus endometrium or myometrium could potentially yield some false-positive results (i.e. genes that are inherently differentially expressed between endometrium and myometrium). To address this issue, genes differentially expressed between endometrium and myometrium (Supplementary Table II) were filtered out from the list of 2941 and 2826 probe sets differentially expressed in adenomyosis versus endometrium and myometrium. As a result, 496 and 566 probe sets (1062 altogether) remained up- or down-regulated in adenomyosis versus endometrium, and 425 and 895 probe sets (1320 altogether) remained up- or down-regulated in adenomyosis versus myometrium. Next, we combined the 1062 and 1320 probe sets, keeping the overlapping 309 probe sets only once in the list, resulting in a total of 2073 probe sets as differentially expressed in adenomyosis versus endometrium and myometrium. Table I lists the top 25 up- and down-regulated genes, common in adenomyosis versus both endometrium and myometrium (sorted by fold change in A/endo).

The majority of the differentially expressed probe sets in uterine fibroids versus myometrium were down-regulated and amounted to 1662 (Supplementary Table II). Transcripts up-regulated in fibroids relative to normal myometrium accounted for the remaining 665 probe sets. The list of top 25 up- or down-regulated genes is shown in Table II. The expression of representative genes with altered expression in adenomyosis and uterine fibroids was validated by TaqMan Q-PCR. The results are shown in Figure 3. These data are consistent with the observations from the microarray data.

To understand major themes and networks that may be embedded in the list of 2073 and 2327 probe sets differentially expressed in adenomyosis or uterine fibroids, respectively, these probe sets were imported into the IPA software. Of 2073 probe sets (adenomyosis), 902 were assigned to networks. There were 48 significant networks in adenomyosis (score > 2), of which 9 were highly significant (score $= 35$, focus genes $= 35$). The number one-ranked network (Figure 4a) is associated with cancer and cell death. Top functions of the other eight highly significant networks (Supplementary Figure 2) are associated with cancer, cell morphology, cell cycle, cellular assembly and organization, cellular movement, DNA replication, recombination and repair, embryonic and tissue development, cell signaling, protein synthesis, molecular transport and protein trafficking. In the case of uterine fibroids, 897 probe sets were eligible for generating networks. There were 47 significant networks (score > 2), of which 10 were highly significant (score $= 35$, focus genes $= 35$). The number one-ranked network is associated with cancer, cell death and renal and urological diseases and is shown in Figure 4b. Top functions of the other nine highly significant networks (Supplementary Figure 2) are associated with cellular development, cell signalling, cell movement, cell-to-cell signalling and interaction, tissue development, cell cycle, cell function and maintenance, cellular assembly and organization, RNA post-transcriptional modification, cell growth and proliferation and immunological diseases.

**Differences and similarities between adenomyosis and uterine fibroids at the molecular level**

One of our primary goals was to identify differences between adenomyosis and uterine fibroids at the molecular level. In both the unsupervised PCA (Figure 1) and the supervised cluster analysis using the 7415 probe sets identified as differentially expressed (Figure 2), all the adenomyosis samples grouped separately from all the uterine fibroid samples. Of the 7415 probe sets, 471 were altered by $>2$-fold between adenomyosis and uterine fibroids (Supplementary Table II). Most of these probe sets (i.e. 315) were up-regulated in uterine fibroids, and the remaining 156 probe sets were up-regulated in adenomyosis. Table III summarizes the top 50 most differentially expressed genes between the two diseases. Purkinje cell protein 4
PCP4) had the highest fold change in expression: \(~17\)-fold higher in uterine fibroids than in adenomyosis, and it was confirmed by TaqMan Q-PCR (Figure 3). About two-thirds of the 471 probe sets \((i.e. 302)\) were also differentially expressed between endometrium and myometrium. This finding is consistent with the fact that adenomyosis originates from endometrium and uterine fibroids from myometrium.

The identification of genes such as metastasis-associated lung adenocarcinoma transcript 1 \((MALAT1)\) (non-coding RNA), paternally expressed 3 \((PEG3)\) and v-maf musculoaponeurotic fibrosarcoma oncogene homolog \((MAF)\) (avian) that are altered both in adenomyosis and in uterine fibroids \((RB1),\) phosphatase and tensin homolog \((PTEN)\) (mutated in multiple advanced cancers 1), B-cell CLL/lymphoma 6 \((BCL6)\) (zinc finger protein 51) and cyclin-dependent kinase inhibitor 2C \((CDKN2C)\) \((p18, inhibits CDK4)\) and the increased expression of cyclin D2 \((CCND2)\), v-rel reticuloendotheliosis viral oncogene homolog \((REL)\) (avian) and SMAD, mothers against DPP homologue 5 \((Drosophila)\) \((SMAD5)\) are examples of genes related to these functions \((data not shown)\).

**Figure 2.** Supervised hierarchical cluster analysis based on analysis of variance (ANOVA)/Tukey/significance analysis of microarrays (SAM) selected probe sets. A total of 7415 probe sets identified as differentially expressed \(see Materials and methods\) was used for two-dimensional \(2D)\) hierarchical clustering. Each row represents an individual probe set, and each column represents a tissue sample. In the dendrogram, the horizontal length of each arm reflects the relatedness of tissue sample clusters. The colour spectrum for the range of expression values is shown at the right.
Table I. List of most up- or down-regulated genes in adenomyosis versus endometrium and myometrium

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<th>Gene ID</th>
<th>Gene description</th>
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<th>A/myo</th>
<th>UF/myo</th>
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<td>11.15</td>
<td>9.68</td>
<td>2.12</td>
<td>nsc</td>
</tr>
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A, adenomyosis; endo, endometrium; myo, myometrium; nsc, no significant change [based on analysis of variance (ANOVA)/Tukey/significance analysis of microarrays (SAM)/2-fold threshold]; UF, uterine fibroid.

Discussion

Microarray analysis is a powerful technique that has been applied recently in the study of several human diseases to reveal underlying molecular abnormalities. The pathobiology of adenomyosis is not well understood; therefore, we decided to characterize this disease at the molecular level. Parrott et al. (2001) investigated experimentally induced adenomyosis of mice using cDNA arrays containing a limited set of genes and identified nerve-growth factor-α, pre-adipocyte factor-1 and insulin-like growth factor II as differentially regulated genes in adenomyosis. Green et al. (2005) also performed cDNA microarray analysis of tamoxifen-induced adenomyosis of mice. However, gene expression profiling of human adenomyosis has not been reported. Therefore, our present study—genome-wide gene expression profiling of human adenomyosis using the Affymetrix U133 Plus 2.0 microarray platform—represents the first comprehensive molecular analysis of human adenomyosis. It also represents the first direct comparison of the gene expression profile of adenomyosis with that of uterine fibroids.

The main objectives of our study were to survey the gene expression patterns in adenomyosis: (i) as compared with endometrium and myometrium to gain insights into the underlying biology of this enigmatic disease and (ii) as compared with uterine fibroids to identify potential diagnostic markers that could help the differential diagnosis of these two common diseases. We compared the gene expression...
of the four tissue types, using two independent algorithms (PCA and ANOVA/Tukey/SAM) to analyse data generated from the microarray experiments and to investigate how samples cluster based on the similarities and differences in their gene expression profiles. All probe sets on the Affymetrix chip were used in the PCA, whereas the hierarchical clustering analysis used a more limited probe set (Figure 2). The result is in agreement with the anatomic origin of these two diseases—i.e. adenomyosis derives from endometrium and uterine fibroids from myometrium.

Uterine tissues (both endometrium and myometrium) are highly sensitive to sex steroids. Notably, the two subclusters within the endometrium cluster correspond to the two phases of the menstrual cycle: subcluster of endo 1, endo 2 and endo 3 samples are at follicular phase, and subcluster of endo 4, endo 5 and endo 6 samples are at luteal phase (Figure 2). There were also two subclusters within the adenomyosis samples (Figure 2); however, they do not correspond to endometrium samples and uterine fibroids with myometrium. This is in agreement with the anatomic origin of these two diseases—i.e. adenomyosis derives from endometrium and uterine fibroids from myometrium.
fact that ectopic endometrial glands within adenomyosis are generally of the basalis type and do not respond to cyclic ovarian hormones (Tamai et al., 2005).

Although secretory differentiation is not typical of adenomyosis, progestational effects in non-gravid uterus occurs in ~30–50% of adenomyotic foci (Ferenczy, 1998). Recently, it has been reported that various adenomyosis foci have quite different hormone responsiveness (Nisolle and Donnez, 1997). Those foci lying directly below the basalis layer of the endometrium are frequently unresponsive. However, deep in the myometrium, both responsive and unresponsive endometrium can be identified, often side-by-side. Women with superficial forms of adenomyosis are usually asymptomatic, whereas deep forms of adenomyosis correlate with symptoms (Ascher et al., 2003). All the donors of the adenomyosis samples included in our study had severe symptoms (requiring surgical removal of the whole uterus), likely due to the presence of deep adenomyosis. Therefore, some of the adenomyosis samples in our study may also undergo secretory changes. Consequently, the two subclusters of adenomyosis samples could correspond to the group of adenomyosis samples that are ‘arrested’ at the follicular phase and to the group of adenomyosis samples containing both hormone responsive and unresponsive endometrium. It is also known that about 50% of adenomyosis patients have co-existing uterine fibroid(s) (Bergeron et al., 2006), and this may also have an effect on the gene expression results. In our study, 6 of the 10 adenomyosis patients (A3, A5, A6, A7, A8 and A10) had also uterine fibroids; however, the samples from these patients did not subcluster separately from the other four adenomyosis patients (A1, A2, A4 and A9) (Figure 2).

In patients with adenomyosis, the basal layer of the endometrium invades the myometrium. Another important feature of the disease is myometrial hyperplasia (increase in cell number) and hypertrophy (increase in cell size). Cellular invasion and uncontrolled cell growth are common characteristics of cancer, and therefore, it is not surprising that cancer- and cell death-related functions dominate the top functional network in adenomyosis (Figure 4a). However, adenomyosis is not a cancer (i.e. does not have metastatic potential), and it has...
been only suggested to be associated with endometrial adenocarcinoma. Notably, this network is centered on the up-regulation of the anti-apoptotic and proto-oncogene molecule **BCL2**. The constant expression of **BCL2** has been suggested previously to promote the endometrial invagination into myometrium (Bergeron *et al.*, 2006). Moreover, we found the pro-apoptotic molecule **BCL2-antagonist of cell death (BAD)** to be down-regulated in adenomyosis. A change of mitochondrial membrane permeability is essential for apoptosis, leading to the translocation of apoptogenic cytochrome *c* and apoptosis-inducing factor into the cytoplasm. The **BCL2** family of proteins regulate cytochrome *c* release and the mitochondrial membrane potential by directly modulating the activity of the voltage-dependent anion channel 1 (VDAC1; down-regulated in adenomyosis) through binding (Shimizu *et al.*, 2000). Protein kinase C-ε (**PRKCE**), implicated in the induction of apoptosis (Thiam *et al.*, 1999), was also down-regulated in adenomyosis. The resulting overall impairments in the apoptotic pathways may likely contribute to the survival of endometrial cells within the myometrium, as well as to myometrial hyperplasia in adenomyosis. In the same network, we found members of the RAS and RASSF family to have altered expression [v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (**KRAS**), up-regulated; neuroblastoma RAS viral (v-ras) oncogene homolog (**NRAS**)) and RAS association (RalGDS/AF-6) domain family 4 (**RASSF4**), down-regulated]. The best-characterized roles of oncogenic RAS proteins are the promotion of dysregulated cell cycle progression and uncontrolled cellular proliferation. However, it is now clear that oncogenic RAS proteins can also effect apoptosis (Cox and Der, 2003) and modulate the activity of apoptotic molecules including BAD and BCL2 (Chang *et al.*, 2003). Consequently, RAS proteins may contribute to the pathogenesis of adenomyosis by interfering with the normal balance of cell cycle, cell

**Figure 4.** Most significant network in adenomyosis and uterine fibroids. Probe sets identified as differentially expressed in adenomyosis (2073) or uterine fibroids (2327) were imported into Ingenuity Pathways Analysis (IPA) software. The most significant functional network in both diseases is shown as (a) cancer- and cell death-related network in adenomyosis (score = 35, focus genes = 35) and (b) cancer-, cell death- and renal and urological disease-related network in uterine fibroids (score = 35, focus genes = 35). Pink colour indicates up-regulation in gene expression, whereas down-regulated genes are shown in green.
Gene expression profile of human adenomyosis

In addition to the top network, there were another eight highly significant networks altered in adenomyosis (Supplementary Figure 1). Central molecules in these networks are \( RB1, \) \( CCND1, \) \( BCL6, \) endothelin 1 (\( EDN1 \)), \( \gamma \)-erb-b2 erythroblastic leukaemia viral oncogene homolog 2 (\( ERBB2 \)), \( PTEN, \) interleukin 15 (\( IL15 \)), insulin receptor (\( INSR \)), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4 (\( SMARCA4 \)), presenilin 1 (\( PSEN1 \)), ras-related C3 botulinum toxin substrate 1 (rho family, small GTP-binding protein Rac1) (\( RAC1 \)), tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, \( \zeta \)-polypeptide (\( YWHAZ \)), hypoxia-inducible factor 1, \( \alpha \)-subunit (\( HIF1A \)) and RAN, member of RAS oncogene family (\( RAN \)). Disturbance in cell death, cell cycle, cellular assembly and organization, cellular movement, DNA replication, recombination and repair and so on, represented by the above genes, indicates that the pathogenesis of adenomyosis is a multi-factorial event.

Previously, several microarray studies have reported gene expression profiles of uterine fibroids versus myometrium (Tsibris et al., 2002; Chegini et al., 2003; Skubitz and Skubitz, 2003; Wang et al., 2003; Catherino et al., 2004; Quade et al., 2004; Arslan et al., 2005; Luo et al., 2005). We also compared the gene expression profiles of uterine fibroids versus myometrium and obtained results that are in agreement with the earlier studies. Moreover, ours is the first study to also perform IPA, and we identified many functional networks (i.e. 47) operating in uterine fibroids, the top network being centred on the up-regulation of cyclin-dependent kinase inhibitor 1A (\( CDKN1A; \) also called as p21 or Cip1) and down-regulation of \( RB1 \). p21 is mainly known as an inhibitor of cell proliferation. However, a number

Figure 4. Continued

![Network Diagram](https://academic.oup.com/molehr/article-abstract/12/12/737/1035683)
of recent studies have pointed out that p21 can also act as an inhibitor of apoptosis, and this may counteract its tumour-suppressive functions as a growth inhibitor (Garret and Tyner, 2002). RB1 is a prototype of tumour suppressors (Schubert et al., 1994): it is absent or mutated in many human tumors, and the reintroduction of wild-type RB1 is able to suppress neoplastic phenotypes through the regulation of the cell cycle. Central molecules in the next nine highly significant networks (Supplementary Figure 2) in uterine fibroids are JUN, BCL6, fibroblast growth factor 2 (FGF2), CCND1, epidermal growth factor receptor [erythroblastic leukaemia viral (v-erb-b) oncogene homolog, avian] (EGFR), EDN1, signal transducer and activator of transcription 1 (STAT1), tumour necrosis factor (ligand) superfamly, member 10 (TNFSF10), hepatocyte growth factor (HGF), Pten, insulin-like growth factor II (IGF-II) and nuclear transcription factor Y, β (NFYB). These networks highlight the potential mechanisms (e.g. alterations in cell cycle, apoptosis, cell growth and proliferation) responsible for the relatively uncontrolled growth of uterine fibroids.

Although there are some overlaps in the genes and functions that are altered in uterine fibroids and adenomyosis, these two diseases have distinct molecular signatures (Figures 1 and 2) with 471 probe sets differentially expressed between them by more than 2-fold. We propose that the list of these probe sets (Table III, Supplementary Table II) represents potential diagnostic markers that may help distinguish these diseases pre-operatively using myometrial biopsies. Doublecortex,
lissencephaly, X-linked (doublecortin) (DCX), calpain 6 (CAPN6) and proteolipid protein 1 (Pelizzaeus–Merzbacher disease, spastic paraplegia 2, uncomplicated) (PLP1) are examples of genes that exhibit high fold changes in their expression between the two diseases; however, owing to heterogeneous expression across the samples, they did not reach statistical significance in our microarray experiment (Table II). The heterogeneous expression of these and other genes across the samples (data not shown) suggests that a combination of several genes may be necessary for achieving sensitivity and specificity in the differential diagnosis of adenomyosis from uterine fibroids. The histological evaluation of myometrial biopsies has already been suggested as a pre-operative diagnostic test for adenomyosis (McCausland, 1992). However, when only one myometrial biopsy was taken for histological evaluation, the sensitivity of this method was low (i.e. 8–18.7%) (Popp et al., 1993). Even 10 specimens from the uterus resulted in only 40–70% sensitivity. The histological evaluation of ultrasonographic-guided myometrial biopsy, coupled with PCR measurement of several of the differentially expressed genes between adenomyosis and uterine fibroids identified in the present study, could increase the sensitivity/specificity of diagnosis. Moreover, of potential importance is the differential expression of IL17B between adenomyosis and uterine fibroids (Figure 3). IL17B is a recently described member of the IL17 family of cytokines and may be implicated in tissue repair and local inflammatory processes (Li et al., 2000; Shi et al., 2000). It has been reported previously to be overexpressed in uterine fibroids versus myometrium and to be expressed only at low levels in normal myometrium as well as in several other normal human tissues (Skubitz and Skubitz, 2003). Secreted molecules, such as IL17B, are good diagnostic marker candidates, as they could potentially be measured by commonly applied diagnostic assays (e.g. ELISA) from easily accessible biological samples (e.g. serum). Experiments are currently underway to determine whether the differences in mRNA levels of IL17B translate into differences in their protein levels in the sera of patients with adenomyosis and uterine fibroids.

In conclusion, this study provides genome-wide gene expression profile of human adenomyosis. Although the full implications and biological significance of the differentially expressed genes and networks are not yet completely understood, they may serve as a platform to further explore relevant mechanisms of pathogenesis and improve the understanding of the molecular basis of adenomyosis. Moreover, this study also highlights similarities and differences in adenomyosis and uterine fibroids at the molecular level and provides a list of potential diagnostic markers that may ameliorate their differential diagnosis.

Acknowledgements
This manuscript is dedicated to the memory of Dr Rafael Mora (School of Medicine, University of Baja California, Mexicali, Mexico).

Supplementary material
Supplementary data are available at http://molehr.oxfordjournals.org/.

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A. Hever et al.


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