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MICROARRAY STUDY OF GENE EXPRESSION IN UTERINE LEIOMYOMA

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Uterine leiomyoma is a most common benign neoplasm in women of reproductive age. It arises from the myometrial compartment of the uterus and may transform in some cases to a malignant phenotype. *Aim:* To identify the genes involved in pathogenesis of uterine leiomyoma. *Methods:* We have studied differential gene expression in matched tissue samples of leiomyoma and normal myometrium from the very same people utilizing a cDNA microarray screening method. We also compared our results with previously published microarray data to identify the overlapping gene alterations. *Results:* Based on this comparison we can divide genes deregulated in our study into two groups. The first group comprises genes that to our knowledge have not been previously reported as deregulated in fibroids: *CLDN1*, *FGF7 (KGF)*, *HNRPM*, *ISOC1*, *MAGEC1 (CT7)*, *MAPK12*, *RFC*, *TIE1*, *TNFRSF21 (DR6)*. The second group consists of genes identified also in previous studies: *CCND1 (BCL1), CDKN1A (P21), CRABP2, FN1* and *SOX4 (EVI16)*. In our study *FN1* was the most up-regulated gene, occupying the place between the myometrium and fibroids ranging from 2.07 to 3.64, depending of the probe molecule used for detection. *Conclusions:* Newly identified genes may be regarded as potential diagnostic or prognostic markers of uterine leiomyoma and thus may be very useful as new therapeutic candidates.

Key Words: **microarray, gene expression, uterine leiomyoma.**

Uterine fibroids (also called leiomyoma) are the most common pathology of the genitourinary system in women of reproductive age with an estimated cumulative incidence as high as 70–80% by age 50 [1]. Leiomyoma consists mainly of smooth muscle cells originating from myometrial smooth muscle, but in contrast to parental tissue it exhibits elevated rates of mitotic activity [2]. Historically, uterine fibroids have not been considered a genetic disease [3]. At present, however, clinical studies are beginning to identify DNA polymorphisms that influence leiomyoma risk. Growth of fibroids is believed to depend on ovarian hormones and some intermediate elements such as cytokines and growth factors through which the ovarian hormones may be exerting their growth-stimulatory effects on leiomyomas [4]. Correspondingly, polymorphisms in the IL-12Rbeta1 and IL-6 genes appear to be related to a higher susceptibility to leiomyoma [5, 6]. The polymorphism of a steroid hormone metabolizing gene, *CYP17*, seems to play a role in increasing risk in Black African women [7].

Until recently the genetic background of leiomyoma has not been studied as thoroughly as that of cancer, mainly due to benign nature of this kind of tumors. In some cases, however, leiomyoma may transform to a malignant phenotype, although this phenomenon is uncommon. Malignant transformation is generally thought to be associated with the changes in the expression of several genes and/or with the sequential acquisition of multiple mutations.

Since the year 2000, several gene array studies have examined the differential gene expression between uterine fibroids and normal myometrium [8–17]. These studies resulted in significantly different gene expression patterns, although some genes belonging to defined developmental pathways have been detected as differentially expressed in several experiments. The variation in the results may be attributed to differences in microarray techniques and data analysis methods used in these studies. Also, the different genetic statuses of the investigated tumors and race/ethnicity of the patients may have an influence. Subtle variations in patient characteristics or laboratory conditions may also dramatically alter microarray results. It is clear that no single gene list from a single study is sufficient to make definite conclusions regarding the role of the differentially expressed genes in tumorigenesis.

In this study, we have compared gene expression in tissues of uterine leiomyoma and normal myometrium taken in matched pairs from eight patients of varying age undergoing surgery for symptomatic fibroids.

MATERIALS AND METHODS

Microarray fabrication. For microarray analysis, genes were selected that were known to be highly associated with the occurrence of various forms of cancer. Selection was based on publicly available data as well as analyses performed with comprehensive microarrays that represented all human genes. The appropriate cDNA library clones were obtained from

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Abbreviations used: CCND1 – cyclin D1; CDC25B – cell division cycle 25 homolog B (S. pombe); CDKN1A – cyclin-dependent kinase inhibitor 1A; CLDN1 – claudin 1; CRABP2 – cellular retinoic acid binding protein 2; DUSP6 – dual specificity phosphatase 6; FGF7 – fibroblast growth factor 7; FN1 – fibronectin 1; HBA2 – hemoglobin, alpha 2; HBB – hemoglobin, beta; HBE1 – hemoglobin, epsilon 1; HNRPM – heterogeneous nuclear ribonucleoprotein M; ISOC1 – isochorismatase domain containing 1; MAGEC1– melanoma antigen family C, 1; MAPK12 – mitogen-activated protein kinase 12; RFC – replication factor C (activator 1) 1; SOX4 – SRY (sex determining region Y)-box 4; TIE1 – tyrosine kinase with immunoglobulin-like and EGF-like domains 1; TNFRSF21 – tumor necrosis factor receptor superfamily, member 21.

the RZPD Resource Center of the German Human Genome Project (http://www.rzpd.de). Since they are part of the IMAGE collection (http://image.llnl.gov), they are cloned in different vectors systems. However, all cDNAs could be PCR-amplified using the universal M13 primers. Amplification reactions were carried out in 96-well microtiter dish plates with amino-modified forward primer M13for (5'-GTT TTC CCA GTC ACG ACG TTG-3') and amino-modified reverse primer M13rev (5'-AGC GGA TAA CAA TTT CAC ACA GG-3'). PCR was done in a total volume of 100 µl with 20 µM of each nucleotide triphosphate, 0.1 µM of each primer, 0.5 U Taq DNA polymerase (Qiagen, Hilden, Germany) and the corresponding PCR-Buffer. Using PTC200 cyclers (MJ Research, USA) inoculation was done by transferring clones from bacteria culture using 96-pin replicator. Initial denaturation was done at 95 °C for 5 min, followed by 40 cycles of 30 s at 92 °C, 30 s at 62 °C (initially) and 2 min at 72 °C, followed by a final step at 72 °C for 10 min. After each cycle the annealing temperature of 62 °C was decreased 0.2 °C per cycle. Quality of PCR products was verified by agarose gel-electrophoresis; most products had a length of 0.5–2.0 kb. They were purified with Multiscreen PCR (Millipore GmbH, Schwalbach, Germany) and resuspended in spotting solution (TeleChem International Inc., Sunnyvale, USA) at a minimum concentration of 300 ng/µl. The PCR products were arrayed onto slides with epoxy surface (Schott Nexterion AG, Jena, Germany), DNA spotting was done with a MicroGrid II arrayer (BioRobotics, Cambridge, UK) using SMP3 pins (TeleChem International Inc., Sunnyvale, USA). Each microarray has 7680 spots divided into 32 blocks, which each containing spots in duplicate. After fabrication the microarrays were treated following the protocols of the manufacturer (Schott Nexterion AG, Jena, Germany). After washing, denaturation at 95 °C for 3 min and blocking, the microarrays were dried with nitrogen.

Patients, sample preparation and hybridization. Eight patients with uterine leiomyoma, who underwent hysterectomy in the Clinic of Odessa State Medical University, Odessa, Ukraine, were included in this study. All patients were informed and gave written consent to participate in the study and to allow their biological samples to be genetically analyzed. Approval for this study was obtained from the Scientific Council of Odessa State Medical University. None of the patients received any hormonal medication 3 months before hysterectomy. Sample pairs of tumor and normal myometrium from the same patients were taken within 10–20 min of extirpation of the uterus, placed in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and then transported to the laboratory, where they were frozen to –80 °C. Snap-frozen surgical samples were ground on dry ice with a mortar and pestle and resuspended in RLT buffer. Total RNA was isolated using the RNEasy Mini Kit (Qiagen, Hilden, Germany). The RNA was analyzed on a LabChip® using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) for quality control. The labeling and hybridization reactions were performed in duplicate. An RNA-pool created by mixing all normal samples was used as reference.

For labeling, 10 µg RNA in a volume of 15 µL were mixed with 5 µL Oligo dT primer (Invitrogen, Carlsbad, CA) before heating at 70 °C for 10 min. After placing the RNA/primer mixture on ice for 5 min, 21 µL cDNA master mix containing 8.5 µL 5 × first strand buffer (Invitrogen, Carlsbad, CA), 3.5 µL DTT (Invitrogen, Carlsbad, CA), 3 µL 10 mM mix of dATP, dTTP and dGTP (Fermentas GmbH, St. Leon Rot, Germany), 2 µL 1 mM d CTP (Invitrogen, Carlsbad, CA), 1 µL RNAseOUT (Invitrogen, Carlsbad, CA), 2 µL Superscript II RT (Invitrogen, Carlsbad, CA) and 2 µL either of Cy5-dCTP (Amersham Bioscience, Freiburg, Germany) for reference RNA; or Cy3-dCTP (Amersham Bioscience, Freiburg, Germany) for tumor or normal myometrium RNA from one patient were added, the contents gently mixed, and the reaction incubated at 42 °C for 1 h. After that 1 µL Superscript II RT (Invitrogen, Carlsbad, CA) was added and the reaction continued at 42 °C for 2 h. The reaction was stopped by heating to 70 °C for 10 min. Then 1 µL RNAse H (Invitrogen, Carlsbad, CA) was added and the mixture incubated at 37 °C for 20 min. The resulting cDNA was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), followed by quantification of the cDNA by spectroscopy using an ND-1000 spectrophotometer (PeqLab Biotechnologie GmbH, Erlangen, Germany). Cy3- and C5-labeled and amplified cDNA was mixed and dried by a SpeedVac concentrator. Pellet was dissolved and resuspended in 5 µL 10 mM EDTA and kept at 95 °C for 5 min. After that 35 µL hybridization buffer #1 (Ambion Inc., Austin, USA) heated at 68 °C in advance was added and the mixture was hybridized under glass coverslips to the DNA chip at 62 °C for 16 h in a humidified chamber water bath.

Detection and data analysis. The hybridized slides were washed in 2 × SSC (300 mM NaCl and 30 mM sodium citrate) + 0.2% SDS for 10 min, then in 2 × SSC for 10 min and finally in 0.2 × SSC for 10 min, and then spun-dried to scanning at room temperature. Fluorescence signals were detected on a ScanArray 5000 confocal laser scanner (Packard, Billerica, USA). Quantification of the signal intensities was done with GenePix Pro 6.0 analysis software (Axon Instruments, Inc., Union City, USA).

Data quality assessment, normalization and correspondence cluster analysis were performed with the MIAME-compatible [18] analysis and data warehouse software package M-CHIPS (Multi-Conditional Hybridization Intensity Processing System, K. Fellenberg, DKFZ, www.mchips.org [19–20]).

Before high-level analysis, data had to be normalized and filtered. Each hybridization was normalized with respect to the gene-wise median of the hybridizations belonging to the control condition. Furthermore, because a sufficient number of non-differential genes was available, normalization factors were computed on the basis of the majority of spots [21]. Genes were defined as being differentially transcribed, if they met

the following criteria: first, the relevant signal intensities on the microarray had to be above background by at least twice the standard deviation of the background signal; second, variation of transcript levels had to be significant according to the highly stringent "min-max separation" criterion [21], which calculates the minimum difference between the respective signal intensities of tumor and normal myometrium, respectively. Cluster analysis was performed with correspondence analysis [22], which is an explorative computational method that visualizes associations between genes and hybridizations in a multi-dimensional space. In array-based transcription analyses it proved to be a very valuable tool for biological data interpretation [20].

RESULTS AND DISCUSSION

In order to find differentially expressed genes, we compared fibroids from all patients with normal myometrium from the same patients. Allowing a false discovery rate of 5% we identified 19 deregulated genes (Table). For genes that were represented on the microarray by more than one probe the smallest and largest values are shown. For example, the gene of fibronectin 1 was represented by seven different probe molecules. The change found between the myometrium and fibroids ranged from 2.07 to 3.64, depending on the probe. No other gene had an up-regulation as strong as this one.

Table. Genes deregulated in leiomyoma relative to matched myometrium

Gene	Gene product	Expression ratio leiomyoma/ normal myometrium	Previous reports
CCND1	Cyclin D1	2.05–2.31	[13, 15]
CDC25B	Cell division cycle 25 homolog B (S. pombe)	1.96	–
CDKN1A	Cyclin-dependent kinase inhibitor 1A	2.03	[9–11]
CLDN1	Claudin 1	2.24	–
CRABP2	Cellular retinoic acid binding protein 2	1.97	[9, 11–13, 23]
DUSP6	Dual specificity phosphatase 6	1.80	[27]
FGF7	Fibroblast growth factor 7	2.21	–
FN1	Fibronectin 1	2.07–3.64	[14,15]
HBA2	Hemoglobin, alpha 2	1.87	–
HBB	Hemoglobin, beta	2.26	–
HBE1	Hemoglobin, epsilon 1	2.29	–
HNRPM	Heterogeneous nuclear ribonucleoprotein M	2.22	–
ISOC1	Isochorismatase domain containing 1	1.96	–
MAGEC1	Melanoma antigen family C, 1	1.96	–
MAPK12	Mitogen-activated protein kinase 12	2.76–2.97	–
RFC	Replication factor C (activator 1) 1	2.19	–
SOX4	SRY (sex determining region Y)-box 4	2.14	[9, 27]
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	2.33–2.97	–
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	2.84	–

We also compared our results with previously published data. Based on this comparison, we can divide genes deregulated in our study into two groups. The first group comprises genes that were also identified in the previous studies: *CCND1, CDKN1A, CRABP2, FN1, SOX4*. The second group consists of genes that to our knowledge have not been previously reported as deregulated in fibroids: *CLDN1, FGF7, HNRPM, ISOC1, MAGEC1, MAPK12, RFC, TIE1, TNFRSF21*.

In agreement with our results, significant over-expression of *CRABP2* (also known as *RBP6*) — encoding retinoic acid binding protein — in uterine leiomyoma was one of the most common observations of gene array studies to date [9, 11–13, 23]. It had also been confirmed at the protein level [24]. Retinoic acid has been shown to play a significant role in the development of uterine fibroids in an animal model. In turn, retinoic acid synthesis and mobilization is affected by estrogen which regulates expression of *CRABP2* and *ALDH1A1* [25].

Our results also confirm the suggestion that the cell cycle-regulating protein cyclin D1 encoded by *CCND1* (*BCL1*) is involved in leiomyoma growth [13, 15]. Cyclin D1 forms a complex with and functions as a regulatory subunit of cyclin-dependent kinases CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Over-expression of this gene alters cell cycle progression and is observed frequently in a variety of tumors. The ERalpha-dependent involvement of cyclin D1 in the growth of leiomyomas during the menstrual cycle has been determined previously by Kovács *et al*. [25]. It is known that the expression of cyclin D1 is increased by estradiol. According to these authors, the *ERalpha* and the *CCND1* expression is elevated in leiomyoma during the menstrual cycle. In menopause, there appears to be a switch from *ERalpha* to *ERbeta* expression in leiomyomas, and the induction of *CCND1* is decreased.

The activity of cyclin-CDK complexes is inhibited by the product of the *CDKN1A* (also known as *P21*, *CIP1*) gene, which thus functions as a regulator of cell cycle progression at G1. The *CDKN1A* gene was also found to be up-regulated in our and several other studies [9–11]. It is known, that the expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. Shime *at al.* [26] made an observation that the tranilast-induced expression of *CDKN1A* and *P53* inhibits proliferative activity of uterine leiomyoma cells. Based on this observation and our own data we suppose that some inhibitory mechanisms may be activated in response to proliferation of leiomyoma and that *CDKN1A* is a marker of such inhibitory processes.

One more up-regulated gene determined by our and previous studies [9, 27] is *SOX4* (*EVI16*). This single exon gene that is highly conserved in vertebrates encodes a member of the SOX (SRY-related HMG-box) family of transcription factors involved in the regulation of embryonic development [28]. The protein also participates in the determination of the cell fate and may function in the apoptosis pathway leading to cell death as well as to tumorigenesis. High levels of *SOX4* expression have been reported in a variety of human cancers, but it is still unclear what role is played by *SOX4* during tumorigenesis. It is regarded as a transforming oncogene in prostate cancer [29] and a contributor to the malignant phenotype of adenoid

cystic carcinoma cells by promoting cell survival [30], while Aaboe et al. [31] propose that strong *SOX4* expression correlates with increased bladder cancer patient survival and effectively drives cells to apoptosis. Over-expression of *SOX4* in leiomyoma cells demands further confirmation by quantitative real-time PCR and immunohistochemistry, and its role in leiomyoma pathogenesis still needs to be determined.

FN1 encodes fibronectin, a glycoprotein binding cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Fibronectins are involved in cell adhesion, cell motility, wound healing, and maintenance of cell shape, embryogenesis, blood coagulation, host defense and metastasis. Abundant expression of fibronectin is detected in leukemic dendritic cells from patients with acute myeloid leukemia [32]; this gene is also up-regulated in papillary thyroid carcinoma [33]. *FN1* is over-expressed in the fibrosarcomas and is highly associated with the higher grade liposarcoma tumors [34]. In our experiment, *FN1* was represented on expression microarray chip by seven probes. This gene had been reported as being significantly up-regulated in fibroids in a previous study by Vanharanta *et al.* [15].

Other probe sets also showed markedly consistent changes: *MAPK12* (fibroid/normal myometrium ratio varying from 2.76 to 2.97) and *TIE1* (2.33–2.97). To our knowledge, these genes have not been previously mentioned as deregulated in leiomyoma. The protein encoded by *MAPK12* functions as a signal transducer during differentiation of myoblasts to myotubes, and in relation to this over-expression of *MAPK12* may be regarded as a marker of leiomyoma proliferation. TIE signaling is involved in multiple steps of the angiogenic remodeling process during development, including destabilization of existing vessels, endothelial cell migration, formation of tubes and their subsequent stabilization by mesenchymal cells. The expression levels of *TIE1* have been shown to correlate with progressive tumor growth and development of metastasis by many types of carcinoma [35]. However, we are the first to report the overexpression of *TIE1* in leiomyoma as compared to normal myometrium. In a recent study by Nakayama *et al.* [35], immunohistochemical staining revealed that about 80% of leiomyomas were positive for TIE1 and other components of the angiopoietin pathway. Coupled together, these observations confirm the hypothesis that the angiopoietin pathway may play an important role in the differentiation of fibroids.

Fibroblast growth factors possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. FGF7 protein levels are markedly increased in the mucosal epithelium of different gastric pathologies, including gastric adenocarcinoma [36]. It has been shown that both human myometrium and leiomyoma express mRNA for *FGF7* (also known as *KGF*) [37]. The elevated *FGF7* expression in leiomyoma detected in our study, together with the known tumorigenic potential of FGF7, points to *FGF7* as a potential target for leiomyoma prevention and treatment.

The protein encoded by *CLDN1* belongs to a family of more then 20 claudin proteins essential for the formation of tight junctions playing crucial roles in control of paracellular transport and in the maintenance of cell polarity. Recent investigations have shown that claudin gene expression is frequently altered in various cancers. It has been hypothesized that changes in or loss of expression of claudins can lead to cellular disorientation and detachment, which is commonly observed in neoplasia. *CLDN1* may support tumor suppressive functions in tissues and its expression appears to be decreased in cancer [38, 39]. Correspondingly, decreased expression of *CLDN1* correlates with short disease-free interval in breast cancer [40]; the *CLDN1* mRNA level in tumour is also considerably lower than that in normal breast tissue [41]. However, *CLDN1* is frequently up-regulated in a large proportion of colorectal carcinomas [42]. This is a first report on over-expression of *CLDN1* in uterine leiomyoma and its role in pathogenesis of this disease. It may be used as a tumor marker and target for the treatment. This needs further investigation, however.

We also observed some changes that contradicted previous results. Quite surprising is the over-expression of several human globin genes observed in our study. The alpha (*HBA*) and beta (*HBB*) loci determine the structure of the 2 types of polypeptide chains in adult hemoglobin, Hb A. The epsilon globin gene (*HBE*) is normally expressed in the embryonic yolk sac. *HBA* and *HBB* genes were reported down-regulated in two previous studies [8, 9]. These conflicting results cannot be explained from the current data and highlight the need to supplement abundant research data.

We conclude that determination of the common molecular events involved in uterine leoimyoma initiation and progression is critical for the development of novel strategies for treatment and prevention of this common category of tumor. DNA microarray analysis has been proven to serve as a useful tool in studying global gene expression in human tumors. However, a careful post-analysis follow-up and validation of microarray experiments is needed. In this pilot study, for the first time we identified several differentially expressed genes between fibroids and normal myometrium. These newly identified genes may be regarded as potential diagnostic or prognostic markers and thus may be very useful as new therapeutic candidates. In our further experiments we will focus on confirming these data.

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АНАЛИЗ ЭКСПРЕССИИ ГЕНОВ ПРИ ЛЕЙОМИОМЕ МАТКИ

Лейомиома матки является одним из наиболее распространенных доброкачественных новообразований женской репродуктивной сферы. В некоторых случаях отмечают злокачественную трансформацию данного новообразования. *Цель:* идентификация генов, вовлеченных в патогенез лейомиомы. *Методы:* проведен анализ дифференциальной экспрессии генов в образцах лейомиомы и нормального миометрия одних и тех же пациентов методом ДНК-биочип-гибридизации и проведено сравнение полученных результатов с данными, опубликованными ранее. *Результаты:* выявлены различия в экспрессии ряда генов, которые можно разделить на две группы. Впервые выявлена повышенная экспрессия генов *CLDN1, FGF7 (KGF), HNRPM, ISOC1, MAGEC1 (CT7), MAPK12, RFC, TIE1* и *TNFRSF21 (DR6)* в ткани лейомиомы по сравнению с нормальным миометрием. Ко второй группе можно отнести гены *CCND1 (BCL1), CDKN1A (P21), CRABP2, FN1* и *SOX4 (EVI16)*, уже упоминавшиеся в связи с патогенезом лейомиомы в ряде предыдущих исследований. Наибольшим изменением уровня экспрессии (в 2,07–3,64 раз в зависимости от зонда) характеризовался ген фибронектина *FN1*. *Выводы:* идентифицированные гены могут рассматриваться в качестве потенциальных диагностических и прогностических маркеров лейомиомы матки.

Ключевые слова: **ДНК-биочип, ген, экспрессия, лейомиома матки.**