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7q deletion mapping and expression profiling in uterine fibroids

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Uterine fibroids are some of the most common tumours of females, but relatively little is known about their molecular basis. Several studies have suggested that deletions on chromosome 7q could have a role in fibroid formation. We analysed 165 sporadic uterine fibroids to define a small 3.2 megabase (Mb) commonly deleted region on 7q22.3–q31.1, flanked by clones AC005070 and AC007567. We also used oligonucleotide microarrays to compare the expression profiles of 10 samples of normal myometrium and 15 fibroids, nine of which displayed 7q-deletions. *Activating transcription factor 3*, *patched homolog (Drosophila)*, *homeo box A5*, *death-associated protein kinase 1*, and *retinoic acid receptor responder 3* were downregulated, and *excision repair crosscomplementing 3*, *transcription factor AP-2 gamma* and *protein kinase C beta 1* were upregulated in fibroids. New pathways were discovered related to fibroid formation. The presence or absence of 7q-deletions did not dramatically affect the global expression pattern of the tumours; changes, however, were observed in genes related to vesicular transport and nucleic acid binding.
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Introduction

Uterine fibroids or leiomyomas are benign tumours arising from the smooth muscle lining of the uterus, the myometrium. They are the most common tumours in women of reproductive age and clinically apparent in at least 25% of females, but some estimates suggest that the proportion of affected individuals might be as high as 77% (Stewart, 2001). Although fibroids are benign, they are a common cause of severe morbidity in the form of abnormal bleeding, pelvic pain or discomfort and reproductive dysfunction. Fibroids are the single most common cause for hysterectomy, which also make them a significant cost for the health care system (Ligon and Morton, 2000; Stewart, 2001).

Even though fibroids are very common, not much is known about their genetic background. Cytogenetic studies have detected chromosomal abnormalities in about 30–50% of fibroids (Pandis *et al*., 1991; Rein *et al*., 1991; Vanni *et al*., 1991). The most common alterations have been deletions on chromosome 7q21–q31, and other abnormalities have involved chromosomes 6, 12 and 14 (Nibert and Heim, 1990). In addition, several allelotyping studies of uterine fibroids have discovered deletions on chromosome 7q21–q31 (Ishwad *et al*., 1995, 1997; van der Heijden *et al*., 1998; Mao *et al*., 1999). All these studies consistently point to a common deleted region around markers D7S518 and D7S471, which according to Knudson's two-hit model suggests a leiomyoma suppressor gene in that region. Tumour-specific point mutations in sporadic fibroids have been discovered in only two genes: *fumarate hydratase* (*FH*), a gene coding the enzyme catalysing the hydration of fumarate to malate in the tricarboxylic acid cycle (Krebs cycle), and *K-RAS* (Hall *et al*., 1997; Lehtonen *et al*., 2004).

DNA microarray analysis has been proven to serve as a useful tool in studying global gene expression in human tumours, and a number of studies have already investigated differences in gene expression levels between normal myometrium and uterine fibroids (Tsibris *et al*., 2002; Wang *et al*., 2003; Weston *et al*., 2003). There are clear differences between the results produced by different microarray experiments on fibroids, although some genes, such as *actin binding LIM protein 1*, *collagen type IV alpha 2*, *early growth response 1*, *GATA-binding protein 2*, *endothelin receptor type A*, and *c-jun*, have been detected as differentially expressed in several studies. The variation in the results is likely due to different microarray techniques used, as well as to differences in data analysis methods (Hoffman *et al*., 2004). The small number of samples in some of the

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studies has also compromised the statistical power to identify differentially expressed genes. In addition, none of the previous studies has taken into account different genetic statuses of the investigated tumours, a factor which is likely to affect their gene expression levels. We have used deletion mapping to further characterize the commonly deleted 7q region in uterine fibroids and to classify samples for expression microarray experiments. Assuming that biologically different tumours also have significantly different mRNA expression profiles we used oligonucleotide microarrays to test whether 7q-deletions define a distinct subgroup of fibroids. In addition, to further characterize the gene expression pattern in uterine fibroids, we compared fibroid gene expression profiles to those of normal myometrium.

Results

Fibroid histology

Frozen sections from the samples subjected to microarray experiments were stained with HE, and sample histology was confirmed. All fibroids were defined as typical leiomyomas.

LOH mapping

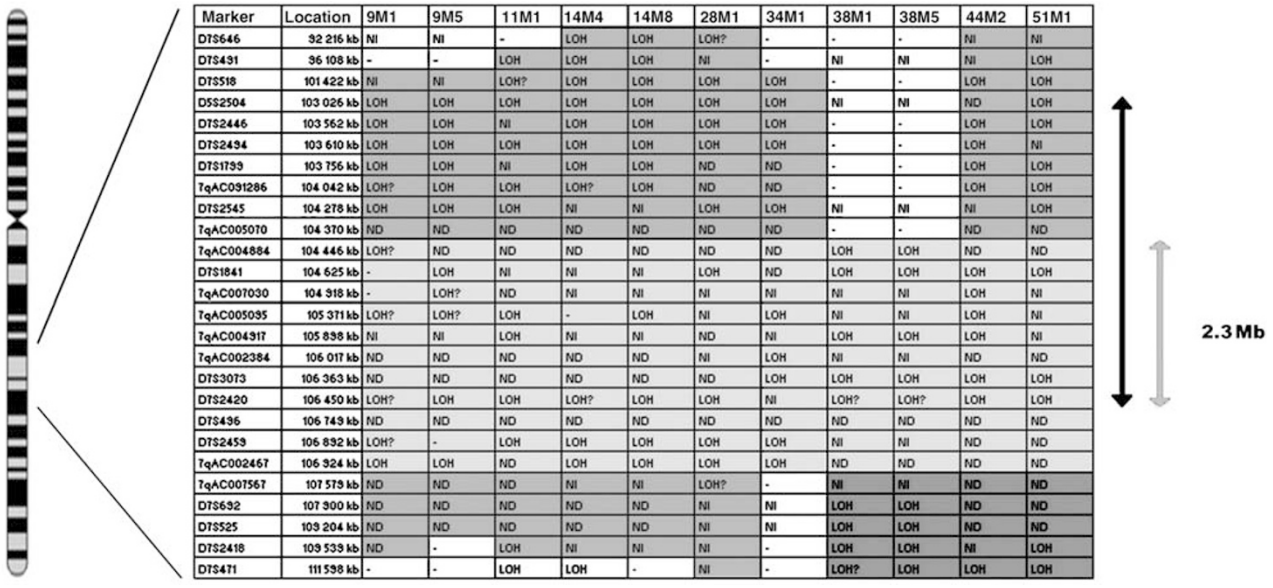
With 25 polymorphic microsatellite markers covering about 20 Mb on chromosome 7q21–q31, 11 fibroids out of 165 (6.7%) showed loss of heterozygosity (LOH) at least with two markers. These fibroids were from eight different patients, that is three patients had two individual tumours that showed LOH on 7q (Figure 1). Although three tumours did not meet the criteria for LOH with all markers in the common region of allelic imbalance (AI), true retention of heterozygosity and thus the possibility of a homozygous deletion was ruled out by a clear trend towards LOH seen in the allele intensity curves. The analysis defined a smallest common region of AI in 7q22.3–q31.1, flanked by markers 7qAC005070 on the centromere side and 7qAC007567 on the telomere side. The results are compatible with a deletion of 3.2 mega bases. It covers at least 22 different transcripts, 20 of which are represented on the HG-U133A chip by 31 different probes (Table 1).

Microarray comparative genomic hybridization (CGH)

Tumours showing LOH on chromosome 7 were further analysed using microarray CGH to confirm that the AI observed using microsatellite markers arises from deletions rather than amplifications. Of 10 samples analysed, eight showed deletion in the region predicted by LOH, while two samples, 34 and 51 m1, showed no deletion. One of the samples showing no predicted loss (51 M1) demonstrated whole chromosome gains of chromosomes 8, 9, 12, 14, and 19. These chromosomal alterations were unique to this sample. Homozygous deletions were not observed in any of the samples.

Gene expression analysis

A total of 25 samples from 11 patients were successfully hybridized on expression microarray chips. There were 10 samples of normal myometrium (N), and the 15



Marker	Location	9M1	9M5	11M1	14M4	14M8	28M1	34M1	38M1	38M5	44M2	51M1
DTS646	92 216 kb	NI	NI	-	LOH	LOH	LOH?	-	-	-	NI	NI
DTS431	96 108 kb	-	-	LOH	LOH	LOH	NI	-	NI	NI	NI	LOH
DTS518	101 422 kb	NI	NI	LOH?	LOH	LOH	LOH	LOH	-	-	LOH	LOH
D5S2504	103 026 kb	LOH	LOH	LOH	LOH	LOH	LOH	LOH	NI	NI	ND	LOH
DTS2446	103 562 kb	LOH	LOH	NI	LOH	LOH	LOH	LOH	-	-	LOH	LOH
DTS2434	103 610 kb	LOH	LOH	LOH	LOH	LOH	LOH	LOH	-	-	LOH	NI
DTS1799	103 756 kb	LOH	LOH	NI	LOH	LOH	ND	ND	-	-	LOH	LOH
7qAC091286	104 042 kb	LOH?	LOH	LOH	LOH?	LOH	ND	ND	-	-	LOH	LOH
DTS2545	104 278 kb	LOH	LOH	LOH	NI	NI	LOH	LOH	NI	NI	NI	LOH
7qAC005070	104 370 kb	ND	ND	ND	ND	ND	ND	ND	-	-	ND	ND
7qAC004884	104 446 kb	LOH?	ND	ND	ND	ND	ND	ND	LOH	LOH	ND	ND
DTS1841	104 625 kb	-	LOH	NI	NI	NI	LOH	ND	LOH	LOH	LOH	LOH
7qAC007030	104 318 kb	-	LOH?	ND	NI	NI	NI	NI	NI	NI	LOH	NI
7qAC005095	105 371 kb	LOH?	LOH?	LOH	-	LOH	NI	LOH	NI	NI	LOH	NI
7qAC004917	105 838 kb	NI	NI	LOH	NI	NI	ND	NI	LOH	LOH	LOH	NI
7qAC002384	106 017 kb	ND	ND	ND	ND	ND	NI	LOH	NI	NI	ND	ND
DTS3073	106 363 kb	ND	ND	ND	ND	ND	ND	LOH	LOH	LOH	LOH	LOH
DTS2420	106 450 kb	LOH?	LOH	LOH	LOH?	LOH	LOH	NI	LOH?	LOH?	LOH	LOH
DTS436	106 749 kb	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DTS2453	106 832 kb	LOH?	-	LOH	LOH	LOH	LOH	LOH	NI	NI	ND	ND
7qAC002467	106 924 kb	LOH	LOH	ND	LOH	LOH	LOH	LOH	ND	ND	ND	ND
7qAC007567	107 579 kb	ND	ND	ND	NI	NI	LOH?	-	NI	NI	ND	ND
DTS632	107 300 kb	ND	ND	ND	ND	ND	NI	NI	LOH	LOH	ND	ND
DTS525	109 204 kb	ND	ND	ND	ND	ND	NI	NI	LOH	LOH	ND	ND
DTS2418	109 539 kb	ND	-	LOH	NI	NI	NI	-	LOH	LOH	NI	LOH
DTS471	111 598 kb	-	-	LOH	LOH	-	NI	-	LOH?	LOH	LOH	LOH

Figure 1 Deletion map of chromosome 7q. NI, noninformative; ND, no data; LOH, loss of heterozygosity; LOH(?), marginal loss of heterozygosity; dash (–), heterozygosity. Scoring of LOH, see Materials and methods. Sample names are shown in the top bar; the first number refers to the patient. Note that patients 9, 14, and 38 had two individual fibroids with a deletion. In the deletion matrix, the darkest grey shaded area highlights a deletion and the lightest grey normal heterozygosity in individual samples. The medium grey shaded colour shows the 3.2 Mb commonly deleted region found in our study. The black double-headed arrow indicates the 5.3 Mb commonly deleted region based on previous reports. The smaller double-headed arrow indicates the 2.3 Mb commonly deleted region based on previous studies combined with new data

Table 1 Transcripts and probes located between clones AC005070 and AC007567

Gene name	*Ref Seq*	*Gene symbol*	*Probe set ID*	*Count*[a]	P-*value*[b]	*Fold change*[c]
SFRS protein kinase 2	NM_003138	SRPK2	203181_x_at	8	0.05	0.26
			203182_s_at	15	0.18	0.83
			214931_s_at	12	0.17	0.87
Hypothetical protein FLJ20485	NM_019042	FLJ20485	218984_at	15	0.49	0.91
Rad50-interacting protein 1	NM_021930	RINT-1	218598_at	15	0.04	0.81
Hypothetical protein MGC33190	NM_152749	MGC33190	214342_at	0		
			214343_s_at	0		
Hypothetical protein FLJ23834	NM_152750	FLJ23834				
Synaptophysin-like protein	NM_006754	SYPL	201259_s_at	15	0.28	0.78
			201260_s_at	15	0.23	0.82
Pre-B-cell colony-enhancing factor 1	NM_005746	PBEF1	217738_at	15	0.75	0.95
			217739_s_at	14	0.63	1.14
Hypothetical protein FLJ36031	NM_175884	FLJ36031				
Phosphoinositide-3-kinase, catalytic, gamma polypeptide	NM_002649	PIK3CG	206369_s_at	0		
			206370_at	0		
Protein kinase, cAMP-dependent, regulatory, type II, beta	NM_002736	PRKAR2B	203680_at	14	0.07	0.69
HMG-box transcription factor 1	NM_012257	HBP1	207361_at	8	0.71	0.79
			209102_s_at	15	0.79	0.95
Component of oligomeric Golgi complex 5	NM_006348	COG5	203629_s_at	15	0.02	0.73
			203630_s_at	15	0.60	0.90
G-protein-coupled receptor 22	NM_005295	GPR22	221288_at	2		
Protein similar to *E. coli* yhdg and *R. capsulatus* nifR3	NM_007016	PP35	205761_s_at	0		
			205762_s_at	3		
B-cell receptor-associated protein 29	NM_018844	BCAP29	205084_at	15	0.54	0.77
Solute carrier family 26, member 4	NM_000441	SLC26A4	206529_x_at	11	0.57	1.08
Cas-Br-M (murine) ecotropic retroviral transforming sequence-like 1	NM_024814	CBLL1	220018_at	11	0.35	1.20
Solute carrier family 26, member 3	NM_000111	SLC26A3	206143_at	1		
Dihydrolipoamide dehydrogenase	NM_000108	DLD	209095_at	15	0.67	0.92
Laminin, beta 1	NM_002291	LAMB1	201505_at	15	0.27	0.79
			211651_s_at	15	0.08	0.73
Laminin, beta 4	AC005048	LAMB4	215516_at	0		
Neuronal cell adhesion molecule	NM_005010	NRCAM	204105_s_at	0		
			216959_x_at	9	0.67	1.08

[a]The number of tumours in which the probe is called present (P). [b]Two-sided heteroscedastic *t*-test between groups N7Q and 7QD calculated only when more than two measurements available in each group. [c]Comparison between groups N7Q and 7QD. Fold change >1 if the expression is higher in 7QD fibroids

tumour samples consisted of two different subgroups: nine fibroids with deletions on chromosome 7q (7QD) and six fibroids without a 7q-deletion (N7Q). An unsupervised hierarchical cluster tree (Figure 2) with all 25 samples was constructed as described in Materials and methods. With the 1057 most differentially expressed probes across the arrays, the samples were divided into two major branches, one mainly consisting of normal myometrium and one of fibroid samples. Tumours with 7q-deletions were not clearly separated from the group N7Q.

Normal myometrium compared to fibroids

To find differentially expressed genes we first compared normal myometrium to all fibroids as one group (7QD + N7Q). Allowing a false discovery rate of 5% we identified 133 differentially expressed sequences, 54 being upregulated (Table 2) and 79 downregulated (Table 3) in fibroids compared to adjacent myometrium. These corresponded to at least 111 different genes as 10 genes were represented by more than one probe set. Different probe sets of each gene showed markedly consistent fold changes, for example, *fibronectin 1* was represented by four probes, the fold changes of which between the myometrium and fibroids ranged from 1.72 to 1.79. Other examples were *tenascin XB* with four probes (fold changes from 0.42 to 0.49), and *nuclear factor I/B* with three probes (fold changes from 0.59 to 0.63).

The most upregulated genes in fibroids were *proteolipid protein 1* (*PLP1*) and *excision repair crosscomplementing 3* (*ERCC3*). They were the only genes that had a fold change greater than 3. On the other hand, the most down-regulated genes in fibroids were *activating transcription factor 3* (*ATF3*), *patched homolog* (*PTCH*), and *baculoviral IAP repeat-containing 3* (*BIRC3*) with fold changes 0.20, 0.28, and 0.32, respectively. Other down-regulated genes were, for example, *growth arrest and DNA-damage-inducible beta* (*GADD45B*), *retinoic acid receptor responder 3* (*RARRES3*), *homeo box A5* (*HOXA5*), *B-cell CLL/lymphoma 6* (*BCL6*), and *death-associated protein kinase 1* (*DAPK1*). Interesting upregulated genes in fibroids were *cyclin D1* (*CCND1*), *phosphodiesterase 8B* (*PDE8B*), *transcription factor AP-2 gamma* (*TFAP2C*), and *protein kinase C beta 1* (*PRKCB1*). The differential expression of two genes were further studied with real-time quantitative polymerase chain reaction (PCR); both *DAPK1* and *RARRES3* showed significant downregulation with *P*-values of 0.019 and 0.038, respectively, thus confirming expression microarray results.

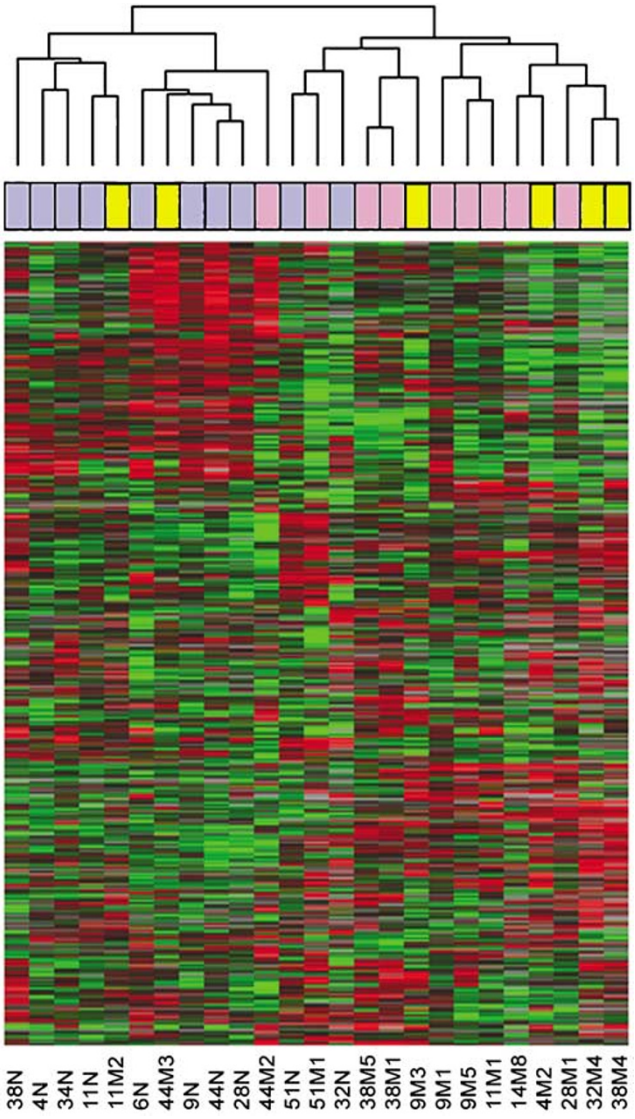


Figure 2 Unsupervised hierarchical cluster tree of all 25 samples. Blue, purple and yellow bars indicate samples of normal myometrium, fibroids with 7q deletions and fibroids without a deletion, respectively. Relative increased and decreased expression of 1057 most differentially expressed genes across the samples is depicted in red and green, respectively. The sample names are shown below the tree

Differentially expressed genes were grouped into biologically meaningful categories according to Gene Ontology (GO) information (Ashburner *et al*., 2000). Of the 111 genes 87 had at least one GO annotation, and they belonged to 515 categories. A functional group enrichment test showed that 40 of the ontology terms represented by more than two genes were enriched with differentially expressed genes (Supplementary Table 1). Among these, however, were several overlapping or nearly identical categories and thus the true number of differentially expressed functional groups was substantially lower. For example, transcription-related genes, 13 of which were downregulated (*ATF3*, *BCL6*, *CITED2*, *EGR1*, *GATA2*, *HOXA5*, *JUN*, *KLF2*, *KLF4*, *MSX1*, *NFIB*, *NR3C1*, *NR4A2*) and four upregulated in fibroids (*SATB2*, *SOX4*, *TFAP2C*, *TRPS1*) were represented in several categories such as Transcription Factor Activity (GO: 0003700), Transcription (GO: 0006350), Transcription Regulator Activity (GO: 0030528), and DNA Binding (GO: 0003677). Additional categories significantly enriched with genes differentially expressed were Response to Stress (GO: 0006950) with 13 genes, three of which were upregulated (*DDB2*, *ERCC3*, *FN1*) and eight downregulated (*APOL3*, *BCL6*, *C1R*, *C1S*, *GADD45B*, *MAP3K5*, *NR3C1*, *NR4A2*, *PDLIM1*, *SMAD7*) and Extracellular Matrix (GO: 0005578) with nine genes upregulated (*COL1A1*, *COL3A1*, *COL4A1*, *COL4A2*, *COL5A2*, *COL9A2*, *LTBP2*, *MFAP2*, *MMP11*) and two downregulated (*LTBP1*, *TNXB*) in fibroids.

Fibroids with 7q-deletions compared to fibroids without deletions

We next compared the expression profiles of 7QD and N7Q fibroids. As the cluster analysis shown in Figure 2 suggested, no gross differences were observed at the level of overall expression profile. At the level of individual genes this was reflected by the fact that the smallest median false discovery rate obtained after permutations was estimated to be $>50\%$. To identify the genes most likely to be differentially expressed between the two groups, we used a two-sided heteroscedastic *t*-test for sorting the probes according to unadjusted *P*-values; the 107 probes with a *P*-value smaller than 0.01 are listed in Supplementary Table 2. Only two of the genes, *CAV2* and *CAPZA2*, are located on the long arm of chromosome 7, but neither of them falls into the small commonly deleted region. We grouped the genes into functional categories and found 22 GO categories enriched with more than two genes differentially expressed (Supplementary Table 3). The most clearly enriched were Nuclear Membrane (GO: 0005635), ER to Golgi Transport (GO: 0006888), Endomembrane System (GO: 0012505), and Nucleic Acid Binding (GO: 0003676). As seen in the normal-to-fibroid comparison, several categories were closely related and thus the real number of functionally enriched groups was smaller than 22.

When looking specifically at the expression of the probes located on the commonly deleted region in 7q, no single candidate tumour suppressor could be identified. The 31 probes represented 20 of the 22 different transcripts in the region, and thus two transcripts were not represented on the chips used. Also, six probes were called absent in all 25 samples, two probes were present only in one sample and one probe only in two samples. In addition, one of the probes representing *PP35* was detected in five normal samples but only in three tumours. This left 13 transcripts out of 22 that were consistently expressed at detectable levels. As Table 1 shows, most of the probes in the deleted region had lower expression in 7QD fibroids relative to N7Q, although only two of them had a *P*-value smaller than 0.05: *component of oligomeric golgi complex 5* (*COG5*, $P=0.02$) and *Rad50-interacting protein 1* (*RINT-1*,

Table 2 Genes upregulated in fibroids compared to normal myometrium

Probe set ID	*Gene title*	*Gene symbol*	*Ref seq*	*Fold change*	q-*value*
210198_s_at	Proteolipid protein 1	PLP1	NM_000533	3.49	0.03
202176_at	Excision repair crosscomplementing 3	ERCC3	NM_000122	3.07	0.02
203878_s_at	Matrix metalloproteinase 11 (stromelysin 3)	MMP11	NM_005940	3	0.04
202421_at	Immunoglobulin superfamily, member 3	IGSF3	NM_001542	2.88	0.01
208712_at	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	NM_001758	2.67	0.03
202016_at	Mesoderm-specific transcript homolog (mouse)	MEST	NM_002402	2.58	0.04
204463_s_at	Endothelin receptor type A	EDNRA	NM_001957	2.57	0.01
202409_at	CDNA clone MGC:52263 IMAGE:4123447, complete cds	—	AK025719	2.54	0.01
219628_at	p53 target zinc-finger protein	WIG1	NM_022470	2.38	0.01
213435_at	SATB family member 2	SATB2	NM_015265	2.37	0.04
209170_s_at	Glycoprotein M6B	GPM6B	NM_005278	2.3	0.01
213228_at	Phosphodiesterase 8B	PDE8B	NM_003719	2.3	0.01
215304_at	Human clone 23948 mRNA sequence	—	U79293	2.26	0.03
37512_at	3-Hydroxysteroid epimerase	RODH	NM_003725	2.01	0.01
213103_at	START domain containing 13	STARD13	NM_052851	2.01	0.03
205286_at	Transcription factor AP-2 gamma	TFAP2C	NM_003222	2	0.01
202589_at	Thymidylate synthetase	TYMS	NM_001071	1.99	0.01
207220_at	Dombrock blood group	DO	NM_021071	1.98	0.01
209685_s_at	Protein kinase C, beta 1	PRKCB1	NM_002738	1.95	0.03
203417_at	Microfibrillar-associated protein 2	MFAP2	NM_002403	1.94	0.01
218330_s_at	Neuron navigator 2	NAV2	NM_145117	1.94	0.01
202998_s_at	Lysyl oxidase-like 2	LOXL2	NM_002318	1.92	0.04
201416_at	SRY (sex-determining region Y)-box 4	SOX4	NM_003107	1.91	0.01
202517_at	Collapsin response mediator protein 1	CRMP1	NM_001313	1.9	0.01
204682_at	Latent transforming growth factor beta binding protein 2	LTBP2	NM_000428	1.89	0.04
206638_at	5-Hydroxytryptamine (serotonin) receptor 2B	HTR2B	NM_000867	1.88	0.04
221004_s_at	Integral membrane protein 2C	ITM2C	NM_030926	1.87	0.01
212353_at	Sulfatase 1	SULF1	NM_015170	1.86	0.04
218502_s_at	Trichorhinophalangeal syndrome I	TRPS1	NM_014112	1.86	0.01
204464_s_at	Endothelin receptor type A	EDNRA	NM_001957	1.8	0.01
216442_x_at	Fibronectin 1	FN1	NM_002026	1.79	0.01
221729_at	Collagen, type V, alpha 2	COL5A2	NM_000393	1.78	0.04
212464_s_at	Fibronectin 1	FN1	NM_002026	1.75	0.01
210495_x_at	Fibronectin 1	FN1	NM_002026	1.75	0.01
205347_s_at	Thymosin, beta, identified in neuroblastoma cells	TMSNB	NM_021992	1.75	0.02
203409_at	Damage-specific DNA binding protein 2, 48kDa	DDB2	NM_000107	1.75	0.01
215558_at	CDNA FLJ10256 fis, clone HEMBB1000870	—	AK001118	1.74	0.04
211980_at	Collagen, type IV, alpha 1	COL4A1	NM_001845	1.73	0.02
216259_at	MRNA; cDNA DKFZp686P12200 (from clone DKFZp686P12200)	—	U85992	1.72	0.04
211719_x_at	Fibronectin 1	FN1	NM_002026	1.72	0.01
221844_x_at	Transcribed sequence with moderate similarity to protein sp:P39194 (*H. sapiens*)	—	AV756161	1.72	0.04
202669_s_at	Ephrin-B2	EFNB2	NM_004093	1.7	0.04
218817_at	Hypothetical protein FLJ22649 similar to signal peptidase SPC22/23	FLJ22649	NM_021928	1.67	0.01
213622_at	Collagen, type IX, alpha 2	COL9A2	NM_001852	1.66	0.04
201417_at	SRY (sex-determining region Y)-box 4	SOX4	NM_003107	1.65	0.01
207813_s_at	Ferredoxin reductase	FDXR	NM_004110	1.65	0.04
202310_s_at	Collagen, type I, alpha 1	COL1A1	NM_000088	1.64	0.04
203834_s_at	Trans-Golgi network protein 2	TGOLN2	NM_006464	1.62	0.04
219983_at	HRAS-like suppressor	HRASLS	NM_020386	1.6	0.01
203903_s_at	Hephaestin	HEPH	NM_014799	1.59	0.01
201852_x_at	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	NM_000090	1.58	0.01
215076_s_at	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	NM_000090	1.57	0.01
204893_s_at	Zinc-finger, FYVE domain containing 9	ZFYVE9	NM_004799	1.51	0.01
211964_at	Collagen, type IV, alpha 2	COL4A2	NM_001846	1.51	0.04

$P = 0.04$). *COG5*, however, had also another probe that did not show as clear a downregulation. The expression of *RINT-1* was further studied by quantitative real-time PCR, but no significant deregulation was detected ($P = 0.75$).

Discussion

In this study we have used deletion mapping of chromosome 7q to identify a small commonly deleted region in uterine fibroids. In addition, we investigated the effects of such deletions on the expression profiles of these tumours. Several reports have proposed a role for the 7q-deletions in leiomyoma formation (Ligon and Morton, 2000), but this is the first time their effects have been studied on a transcriptome-wide level. Also, we have evaluated the changes in expression profiles between fibroids and the adjacent myometrium. Although this has been investigated before, the small number of samples has clearly compromised the power of the previous

Table 3 Genes downregulated in fibroids compared to normal myometrium

Probe set ID	*Gene title*	*Gene symbol*	*Ref seq*	*Fold change*	q-*value*
202672_s_at	Activating transcription factor 3	ATF3	NM_001674	0.20	0.01
209815_at	Patched homolog (*Drosophila*)	PTCH	NM_000264	0.28	0.01
210538_s_at	Baculoviral IAP repeat-containing 3	BIRC3	NM_001165	0.32	0.01
205932_s_at	msh homeo box homolog 1 (*Drosophila*)	MSX1	NM_002448	0.36	0.01
219607_s_at	Membrane-spanning 4-domains, subfamily A, member 4	MS4A4A	NM_016650	0.38	0.04
209357_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	NM_006079	0.38	0.01
204719_at	ATP-binding cassette, subfamily A (ABC1), member 8	ABCA8	NM_007168	0.38	0.01
200965_s_at	Actin-binding LIM protein 1	ABLIM1	NM_002313	0.38	0.01
203423_at	Retinol-binding protein 1, cellular	RBP1	NM_002899	0.39	0.01
201693_s_at	Early growth response 1	EGR1	NM_001964	0.39	0.02
209613_s_at	Alcohol dehydrogenase IB (class I), beta polypeptide	ADH1B	NM_000668	0.39	0.02
219993_at	SRY (sex-determining region Y)-box 17	SOX17	NM_022454	0.40	0.03
208623_s_at	Villin 2 (ezrin)	VIL2	NM_003379	0.41	0.01
213451_x_at	Tenascin XB	TNXB	NM_019105	0.42	0.01
216333_x_at	Tenascin XB	TNXB	NM_019105	0.42	0.01
36711_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	NM_012323	0.44	0.03
206093_x_at	Tenascin XB	TNXB	NM_019105	0.44	0.01
214012_at	Type 1 tumour necrosis factor receptor shedding aminopeptidase regulator	ARTS-1	NM_016442	0.44	0.03
209343_at	EF hand domain containing 1	EFHD1	NM_025202	0.45	0.01
219371_s_at	Kruppel-like factor 2 (lung)	KLF2	NM_016270	0.45	0.01
202241_at	Tribbles homolog 1 (*Drosophila*)	TRIB1	NM_025195	0.46	0.01
207574_s_at	Growth arrest and DNA-damage-inducible, beta	GADD45B	NM_015675	0.46	0.01
221841_s_at	Kruppel-like factor 4 (gut)	KLF4	NM_004235	0.47	0.03
218499_at	Mst3 and SOK1-related kinase	MST4	NM_016542	0.47	0.01
201694_s_at	Early growth response 1	EGR1	NM_001964	0.47	0.01
203917_at	Coxsackie virus and adenovirus receptor	CXADR	NM_001338	0.48	0.01
209710_at	GATA-binding protein 2	GATA2	NM_002050	0.49	0.01
216248_s_at	Nuclear receptor subfamily 4, group A, member 2	NR4A2	NM_006186	0.49	0.02
208609_s_at	Tenascin XB	TNXB	NM_019105	0.49	0.01
222303_at	Transcribed sequences	—	NM_005239	0.50	0.01
206707_x_at	Chromosome 6 open reading frame 32	C6orf32	NM_015864	0.50	0.03
203695_s_at	Deafness, autosomal dominant 5	DFNA5	NM_004403	0.53	0.01
210831_s_at	Prostaglandin E receptor 3 (subtype EP3)	PTGER3	NM_000957	0.53	0.02
207980_s_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	NM_006079	0.53	0.03
202081_at	Immediate early response 2	IER2	NM_004907	0.54	0.02
213258_at	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	TFPI	NM_006287	0.54	0.01
205752_s_at	Glutathione S-transferase M5	GSTM5	NM_000851	0.55	0.01
205111_s_at	Phospholipase C, epsilon 1	PLCE1	NM_016341	0.56	0.04
209211_at	Kruppel-like factor 5 (intestinal)	KLF5	NM_001730	0.57	0.03
209708_at	Monooxygenase, DBH-like 1	MOXD1	NM_015529	0.57	0.03
209894_at	Leptin receptor	LEPR	NM_002303	0.57	0.01
203571_s_at	Chromosome 10 open reading frame 116	C10orf116	NM_006829	0.57	0.01
212706_at	RAS p21 protein activator 4	RASA4	NM_032958	0.58	0.03
203140_at	B-cell CLL/lymphoma 6 (zinc-finger protein 51)	BCL6	NM_001706	0.58	0.01
213933_at	MRNA; cDNA DKFZp586M0723 (from clone DKFZp586M0723)	—	AL031429	0.59	0.03
213281_at	v-*jun* sarcoma virus 17 oncogene homolog (avian)	JUN	NM_002228	0.59	0.04
213844_at	Homeo box A5	HOXA5	NM_019102	0.59	0.04
209290_s_at	Nuclear factor I/B	NFIB	NM_005596	0.59	0.03
208747_s_at	Complement component 1, s subcomponent	C1S	NM_001734	0.60	0.03
212642_s_at	Human immunodeficiency virus type I enhancer binding protein 2	HIVEP2	NM_006734	0.60	0.01
212977_at	Chemokine orphan receptor 1	CMKOR1	NM_020311	0.60	0.02
212914_at	Chromobox homolog 7	CBX7	NM_175709	0.60	0.03
222150_s_at	Hypothetical protein LOC54103	LOC54103	AK026696	0.61	0.03
209496_at	Retinoic acid receptor responder (tazarotene induced) 2	RARRES2	NM_002889	0.61	0.03
205325_at	Phytanoyl-CoA hydroxylase-interacting protein	PHYHIP	NM_014759	0.61	0.01
204005_s_at	PRKC, apoptosis, WT1, regulator	PAWR	NM_002583	0.61	0.04
209289_at	Nuclear factor I/B	NFIB	NM_005596	0.61	0.03
210832_x_at	Prostaglandin E receptor 3 (subtype EP3)	PTGER3	NM_000957	0.62	0.04
200800_s_at	Heat shock 70kDa protein 1A	HSPA1A	NM_005345	0.62	0.04
219777_at	Human immune-associated nucleotide 2	hIAN2	NM_024711	0.62	0.03
216321_s_at	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	NR3C1	NM_000176	0.62	0.01
221087_s_at	Apolipoprotein L, 3	APOL3	NM_014349	0.63	0.01
213029_at	Nuclear factor I/B	NFIB	NM_005596	0.63	0.03
203837_at	Mitogen-activated protein kinase kinase kinase 5	MAP3K5	NM_005923	0.63	0.04

Table 3 (*continued*)

Probe set ID	*Gene title*	*Gene symbol*	*Ref seq*	*Fold change*	q-*value*
219157_at	Kelch-like 2, Mayven (*Drosophila*)	KLHL2	NM_007246	0.63	0.01
204790_at	SMAD, mothers against DPP homolog 7 (*Drosophila*)	SMAD7	NM_005904	0.63	0.03
212067_s_at	Complement component 1, r subcomponent	C1R	NM_001733	0.64	0.04
204070_at	Retinoic acid receptor responder (tazarotene induced) 3	RARRES3	NM_004585	0.64	0.01
203139_at	Death-associated protein kinase 1	DAPK1	NM_004938	0.64	0.01
211675_s_at	I-mfa domain-containing protein	HIC	NM_199072	0.64	0.01
219549_s_at	Reticulon 3	RTN3	NM_006054	0.65	0.01
208690_s_at	PDZ and LIM domain 1 (elfin)	PDLIM1	NM_020992	0.65	0.04
200790_at	Ornithine decarboxylase 1	ODC1	NM_002539	0.65	0.02
202729_s_at	Latent transforming growth factor beta binding protein 1	LTBP1	NM_000627	0.65	0.03
217738_at	Pre-B-cell colony-enhancing factor 1	PBEF1	NM_005746	0.65	0.03
200632_s_at	N-myc downstream regulated gene 1	NDRG1	NM_006096	0.66	0.01
217739_s_at	Pre-B-cell colony-enhancing factor 1	PBEF1	NM_005746	0.66	0.01
202908_at	Wolfram syndrome 1 (wolframin)	WFS1	NM_006005	0.66	0.03
201753_s_at	Adducin 3 (gamma)	ADD3	NM_016824	0.66	0.04

studies, and thus made it evident that further efforts to evaluate fibroid gene expression were necessary.

The smallest commonly deleted region defined by LOH analysis was delimited by markers 7qAC005070 and 7qAC007567, a region of 3.2 Mb. Further analysis with microarray CGH confirmed that most of the changes were indeed deletions, not amplifications, but no homozygous deletions were observed. Also, the lack of chromosomal deletions in two tumours showing LOH indicates that mitotic recombination, rather than deletion, may be the cause of LOH in some fibroids (Gupta *et al*., 1997; Blackburn *et al*., 2004). Since the previous deletion mapping efforts on fibroids were conducted before the final version of the human genetic map was available, some of the marker information has changed (Ishwad *et al*., 1995, 1997; van der Heijden *et al*., 1998; Mao *et al*., 1999). In particular, the results by van der Heijden *et al*. rely critically on marker D7S501, the published reverse primer of which does not have a perfect match on the 7q22.3 target region in the latest version (v29.35b) of Ensembl (www.ensembl.org) genome browser, thus making the assessment of LOH in our experience nonreproducible (data not shown). When this and the possibility of a small homozygous deletion as the explanation for retention of heterozygosity (Cairns *et al*., 1994; Ishwad *et al*., 1997) are taken into account, the former smallest commonly deleted region was 5.3 Mb and flanked by markers D7S518 and D7S496. Our data further reduces the commonly deleted region to be flanked by markers 7qAC005070 and D7S496, a region of only 2.3 Mb.

Microarray analyses revealed several genes that to our knowledge have not been previously reported as deregulated in fibroids; for example, *ERCC3*, *TFAP2C*, and *PRKCB1* were upregulated, and *PTCH*, *GADD45B*, *HOXA5*, *DAPK1*, and *RARRES3* were downregulated in fibroids. To test the reproducibility of the results with another method, we analysed *DAPK1* and *RARRES3* expression with quantitative real-time PCR; in both cases the expression microarray results were strongly supported. We also observed some changes that contradicted previous results. For example *fibronectin 1*, which in our study was significantly upregulated in fibroids with four independent probe sets, has been reported as downregulated in fibroids in a microarray study containing only six fibroid samples (Chegini *et al*., 2003). The changes in the expression profiles observed between normal myometrium and fibroid tissue also confirmed some previously reported results (Kovacs *et al*., 2001; Tsibris *et al*., 2002; Ahn *et al*., 2003; Chegini *et al*., 2003; Wang *et al*., 2003; Weston *et al*., 2003). For example, *CCND1*, *endothelin receptor type A* (*EDNRA*), *collagen type III alpha 1* (*COL3A1*), and *collagen type IV alpha 2* (*COL4A2*) were upregulated, and *early growth response 1* (*EGR1*), *complement component 1r* (*C1R*), *actin binding LIM protein 1* (*ABLIM1*), *v-jun sarcoma virus 17 oncogene homolog* (*JUN*), and *B-cell CLL/lymphoma 6* (*BCL6*) were downregulated in fibroids.

Although definitive conclusions regarding the role of the differentially expressed genes in tumorigenesis are very difficult to make, based on the current literature it is tempting to hypothesize that some of them could be directly involved in fibroid formation and growth. For example, death-associated protein kinase 1 (DAPK1) is a serine/threonine kinase that is associated with the cytoskeleton, and its expression is reduced in several types of human cancer, mainly due to promoter hypermethylation. This kinase mediates cell death in response to various stimuli such as TNF-α, TGF-β and detachment from the extracellular matrix (Gozuacik and Kimchi, 2004). One study showed that DAP kinase activates a p19ARF/p53-dependent apoptotic checkpoint and thus counteracts oncogene-induced transformation (Raveh *et al*., 2001). In this study, we have detected reduced DAPK1 expression in fibroids, which could facilitate fibroid cells to avoid cell death and thus promote tumour growth. Another interesting gene downregulated in fibroids was *RARRES3*. It is a tumour suppressor candidate and it mediates growth suppressive effects of retinoids (DiSepio *et al*., 1998). Notably, also other genes involved in the effects of retionoids were downregulated in fibroids, for example, *retinoic acid receptor responder 2* (*RARRES2*) and *retinol binding protein 1* (*RBP1*).

To further understand the biological meaning of the differentially expressed genes at the level of functional categories, a functional group enrichment test was implemented. It revealed that genes related to extracellular matrix, transcription, and cellular stress responses were deregulated in fibroids. Collagen has been shown to directly stimulate smooth muscle cell growth (Herz *et al*., 2003) and regulate cell migration (Parameswaran *et al*., 2004). Increased expression of collagens and other extracellular matrix proteins could thus have an important role in fibroid growth. Of the 13 differentially expressed genes related to stress responses, only three were upregulated in fibroids. Two of them, *DDB2* and *ERCC3*, are both involved in nucleotide excision repair (Itoh *et al*., 2004; Terashita *et al*., 2004).

Previously, it has been shown that commonly deleted genomic regions in tumours can harbour tumour suppressor genes (Li *et al*., 1997). Also, it has been shown, that mutations of a single tumour suppressor gene can significantly alter the global expression pattern of a tumour (Mori *et al*., 2003). We hypothesized that fibroids with deletions on 7q form a distinct subgroup of fibroids due to the loss of a putative tumour suppressor gene on 7q, and this would be reflected in the global gene expression profile of the respective fibroids. However, when comparing the gene expression of nine tumours with 7q deletions to six tumours without deletions, a method based on false discovery rate estimation (Tusher *et al*., 2001) did not find differentially expressed genes with an acceptable confidence. To reduce the stringency of the correction for multiple tests, an unadjusted *t*-test was used, and this detected 107 sequences differentially expressed with a *P*-value smaller than 0.01. Among these there were two genes that map to chromosome 7q, although neither of them locates in the small commonly deleted region. A more specific look at the genes in the deleted region revealed that most of the probes were indeed downregulated in fibroids with 7q-deletions, although only two, *RINT-1* and *COG5*, had *P*-values smaller than 0.05. Neither of these, however, qualified as a differentially expressed gene when corrected for the estimated rate of false discoveries, and in accordance with this, no downregulation of *RINT-1* was observed in further studies with quantitative real-time PCR. Thus our analysis could not unequivocally identify a single candidate tumour suppressor gene in the commonly deleted region on 7q.

These results suggest that N7Q and 7QD fibroids are biologically relatively similar. It is possible that the deletions interfere with a genetic pathway that is altered in the rest of fibroids through a different mechanism, for example, promoter hypermethylation of the putative leiomyoma suppressor on 7q, and this could obscure the differences between the two groups of fibroids. Furthermore, different deletions could target different leiomyoma suppressor genes, or the suppressor could be haploinsufficient, which would greatly complicate the identification of the gene. Yet another possibility is that the tumour suppressor is an RNA gene that does not code proteins (Palatnik *et al*., 2003), which also would make the identification of the tumour suppressor more difficult. It is also possible that the deletions do not have a biologically significant role in fibroid formation. This explanation, however, does not appear appealing, since the deletions have been independently reported to map into a consistent region on 7q21–q31. In addition, only 13 of the 22 commonly deleted transcripts were consistently expressed at detectable levels in our microarray experiment. This leaves 10 additional candidate genes that could be downregulated in fibroids with 7q-deletions, but that would have escaped our analyses. Although many of these are relatively poorly characterized, for example *FLJ23834* (NM_152750) is an interesting candidate as it contains cadherin domains and is homologous to *Drosophila fat* tumour suppressor.

In conclusion, our results presented in this report suggest that 7q-deletions do not substantially modify the global gene expression profiles in uterine fibroids, although it still remains possible that the deletions have an important role in their pathogenesis. Our mapping effort further reduced the commonly deleted region on chromosome 7q22–q31 to only 2.3 Mb, and the expression microarray analysis gave novel information regarding the changes in global gene expression pattern caused by the deletions. This will be valuable when further experiments are conducted to test whether one or several of the deleted genes truly act as a tumour suppressor. In addition, significant biological insight was gained from the identification of multiple differentially expressed genes between fibroids and normal myometrium for the first time. These results suggest new pathways involved in the pathogenesis of uterine fibroids and provide a platform for the formulation and testing of new, more specifically targeted hypotheses regarding their biology.

Materials and methods

Tumour materials

In all, 165 fibroid specimens and corresponding normal myometrium from 51 anonymous and unselected Finnish patients were collected at The Helsinki University Central Hospital with the approval of the hospital ethics review committee. The samples were snap frozen in liquid nitrogen and then stored at -80°C. Frozen section slides from the tumours for microarray experiment were HE stained and the diagnosis was confirmed. DNA extractions were performed with standard procedures. For the microarrays, at least 30 μg of total RNA were extracted from each sample with the Trizol® reagent (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA) and an RNA clean-up with RNeasy® mini columns (Qiagen Ltd, West Sussex, UK) was performed according to the manufacture's instructions. An aliquot was subjected to RNA 6000 Nano Chip (Agilent Tecnologies, Waldbronn, Germany) analysis to verify RNA integrity.

Analysis of LOH

A total of 25 microsatellite markers on chromosome 7q were used to study LOH in all 165 sporadic tumours (Supplementary Table 4). Fluorescence-labelled primers (Sigma-Genosys, Cambridgehire, UK) were used to amplify matching normal and tumour DNA with PCR. The PCR products were detected with ABI377 sequencer and analysed with Genotyper 2.5.2

software (Applied Biosystems, Foster City, CA, USA). LOH was scored by calculating the intensity ratio of the constitutional alleles as described before (Canzian *et al*., 1996). Intensity ratio $L<0.60$ or $L>1.67$ was called LOH, $0.60<L<0.75$ or $1.33<L<1.67$ was called marginal LOH and $0.75<L<1.33$ was called normal heterozygosity.

Array-CGH analysis

The array construction and hybridization were performed as described (Fiegler *et al*., 2003; Douglas *et al*., 2004). In brief, the array was prepared from DOP-PCR amplified DNA from 3452 large insert genomic clones at an average spacing of about 1 Mb throughout the genome. DNA from each of the fibroids was labelled with Cy5-dCTP. DNA from a pool of normal individuals was used as a control and labelled with Cy3-dCTP. The test and normal DNAs were cohybridized to the arrays together with herring sperm and CotI DNA and washed. The array was scanned using a confocal scanner (Perkin Elmer) and Spot (Jain *et al*., 2002) software was used to process the images. After rejecting poorly hybridized arrays by visual inspection of scans and correcting for background, the $\log_2$ ratio of the fluorescence intensities of test (tumour, T) to reference (normal, N) was calculated, after normalization to the remainder of the genome in that tumour. Standard deviations of these ratios were calculated genome-wide. $\log_2$ T:N ratios of >3 were taken to indicate gain and ratios of <-3 were scored as loss. Copy number changes of the sex chromosomes were not analysed.

Synthesis of cDNA and biotin-labelled cRNA

The method for synthesising double-stranded cDNA has been described previously (Mahadevappa and Warrington, 1999). In all, 8 μg of RNA were reverse-transcribed to cDNAs using SuperScript™ Double-Stranded cDNA Syntehesis Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) and T7-(dT)24 primers (5′-GGCCAGTGAATTGTAATACGACTCACTA TAGGGAGGCGG-(dT)24-3′, 100 pmol/μl) in a reaction volume of 20 μl according to the method suggested by the manufacturer, except that the incubation temperature was 42°C. The second-strand cDNAs were then synthesized in a total volume of 150 μl, also according to the manufacturer's protocol, and were purified using Phase Lock Gel™ Heavy 1.5 ml tubes (Eppendorf AG, Hamburg, Germany) and phenol:chloroform premixed with isoamyl alchohol (Amresco, Solon, OH, USA) followed by ethanol precipitation according to the instructions in Affymetrix GeneChip© Expression Analysis Technical Manual (Affymetrix Inc., Santa Clara, CA, USA). BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Diagnostics Inc, Farmingdale, NY, USA) was used to synthesize biotin-labelled cRNA from the purified double-stranded cDNA according to the manufacturer's instructions. The labelled cRNA was then purified with RNeasy® mini columns (Qiagen Ltd).

Fragmentation, array hybridization, and scanning

Labelled cRNA was fragmented by incubation at 94°C for 35 min in the presence of 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate. The hybridization solution consisted of 15 μg fragmented cRNA and 0.1 mg/mL herring sperm DNA in MES buffer (containing 100 mM MES, 1 M Na+, 20 mM EDTA, and 0.01% Tween 20). The samples were hybridized on GeneChip® HG-U133A oligonucleotide chips (Affymetrix) at 45°C for 16 h. Subsequent washing and staining of the arrays were carried out with Fluidics Station 450 (Affymetrix) as outlined in the GeneChip® Expression Technical Manual on GeneChip® probe arrays (Affymetrix). The probe arrays were scanned (multiple image scan) using the GeneChip® System GA2500 Scanner (Affymetrix).

Expression data analysis

MAS 5.0 software (Affymetrix) was used to analyse the scanned image and to obtain quantitative information, and the detection calls were determined using cutoff values $\alpha_1=0.05$ and $\alpha_2=0.065$. Subsequently, the data were loaded into dChip 1.3 (Li and Wong, 2001) where it was normalized using the array with mean intensity as the base line. Small values were truncated to 1 and PM/MM difference model was used to check for single, probe and array outliers. Image spikes were treated as outliers as well. Then the expression values of all genes called marginal or present at least in 50% of the samples were imported to Cluster (Eisen *et al*., 1998), where the data were $\log_2$ transformed and arrays were centred to the median.

For unsupervised clustering, gene vectors with s.d. <0.6 were filtered out resulting in a list of 1057 genes that were the most differentially expressed across the samples. Subsequently, the arrays were organized with self-organizing maps, and hierarchically clustered using Pearson's correlation coefficient as the similarity metric. The cluster tree image was created with TreeView 1.60 software (Eisen *et al*., 1998). Differentially expressed genes were detected using Significance Analysis of Microarrays (SAM) software (Tusher *et al*., 2001). In all SAM analyses missing values were imputed with K-Nearest Neighbours Imputer (10 neighbours) and a two-class test for unpaired data was used with 5000 permutations. For the myometrium – fibroid comparison, a median false discovery rate of $<5\%$ was obtained with delta-value 0.8 and fold change $\geqslant 1.5$. To analyse differentially expressed genes in biologically significant subgroups, functional group enrichment analysis was performed with GoMiner (Zeeberg *et al*., 2003). GoMiner uses the two-sided Fisher's exact test to calculate *P*-values for GO categories (Ashburner *et al*., 2000).

Quantitative real-time PCR

The relative *DAPK1*, *RARRES3*, and *RINT-1* expression levels were determined by quantitative real-time PCR. *TATA-box binding protein* (*TBP*) was used as an endogenous control as its expression did not vary significantly between fibroids and myometrium in microarray analysis. cDNA was synthesized from total-RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) in the manufacturer's buffer containing 1 mmol/l of dNTP, 40 U of RNase inhibitor (Promega), and 300 ng of random hexamer primers. The reactions took place at 42°C for 50 min, followed by 95°C for 10 min and 4°C for 5 min. The cDNA was amplified in an ABI PRISM SDS5700 sequencing detector system (Applied Biosystems) using TaqMan® Gene Expression Assays (Applied Biosystems) for all genes according to manufacturer's instructions. All reactions were carried out in triplicate. The mean C_t value of *TBP* was subtracted from the mean C_t values of *DAPK1*, *RARRES3*, and *RINT-1* to obtain a dC_t value. The dC_t values for each sample were scaled to obtain a ddC_t value by subtracting the highest dC_t value of each gene from all dC_t values of the respective gene. Relative expression was calculated using the Equation 2^{-ddC_t} for *DAPK1*, *RARRES3*, and *RINT-1*. A two-sided heteroscedastic *t*-test was used two compare the mean *DAPK1* and *RARRES3* expression of normal myometrium and fibroids, and the mean *RINT-1* expression of groups N7Q and 7QD.

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