

# Insulin-like Growth Factors II exon 9 and E-cadherin-Pml I but not Myeloperoxidase Promoter-463, Urokinase-ApaL I nor Xeroderma Pigmentosum Polymorphisms Are Associated with Higher Susceptibility to Leiomyoma

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**Abstract.** Objectives: To investigate the roles of insulin-like growth factor II (IGF2), myeloperoxidase (MPO), E-cadherin (CDH1), urokinase and xeroderma pigmentosum group A and D (XPA, XPD) polymorphisms upon leiomyoma susceptibility. Study Design: Women were divided into: group 1, leiomyoma (n=158); group 2, non-leiomyoma (n=156). Polymorphisms (IGF2 exon 9\*A/G, MPO-463\*A/G, CDH1-Pml I, urokinase-ApaL, XPA\*A-23G, XPD\*Lys751Gln) were amplified by polymerase chain reaction and detected by electrophoresis after restriction enzyme digestion. Genotype and allelic frequencies were compared between both groups. Results: Associations between leiomyoma with IGF2 and CDH1 polymorphism exist. Proportions of IGF2 exon 9\*AA/AG/GG in and CDH1\* CC/CT/TT in the groups were: group 1, 38/39.2/22.8% and 27.8/66.5/5.7%; group 2, 22.4/53.9/23.7% and 21.2/64.1/14.7. MPO, urokinase, XPA and XPD in both groups were non-significantly different. Proportions of MPO\*AA/AG/GG, urokinase\*CC/CT/TT, XPA\*AA/AG/GG and XPD\*AA/AC/CC were: group 1: 1.9/23.4/74.7%, 0.6/7/92.4%,

20.9/55.1/24%, 85.4/14.6/0%; group 2: 3.8/24.4/71.8%, 1.3/4.5/94.2%, 22.4/53.9/23.7%, 84.6/15.4/0%. Conclusion: IGF2\*A allele and CDH1\*C allele were correlated with leiomyoma susceptibility, which may be associated with leiomyoma development. MPO, urokinase, XPA and XPD polymorphisms are not related to leiomyoma susceptibilities.

Leiomyoma, the most common benign uterine neoplasma, occurs in around one in four women during their lifetime (1). The factors involved in the initiation and growth of leiomyoma remain unclear. Traditionally, oestrogen has been considered to be the major promoter of leiomyoma growth (2). However, formation of leiomyoma is viewed as a multi-step processes (3). The neoplastic transformation of myometrium to leiomyoma likely involves somatic mutations of normal myometrium and the complex interactions of sex steroids, cytokines, local growth factors, DNA mutations and the dysfunction of the apoptosis system (4).

Growth factors and their receptors play important roles in the pathogenesis of leiomyoma (5). Some polypeptide hormones, such as insulin-like growth factor II (IGF2), have been reported to influence the growth of leiomyoma (6). IGF2 could preferentially promote the growth of leiomyoma cells compared to normal myometrial cells (6). Hyperexpression of IGF2 and increased production of IGF2 peptide could act as autocrine growth factors or co-carcinogens (6).

Genomic instabilities of tumour suppressor genes play major roles in the development and progression of various tumours (7). The cadherin complex regulates cellular adhesion and motility, which functions as an invasion suppressor system. E (epithelial) cadherin (CDH1), a

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Table I. The primer sequences and PCR conditions for IGF2, MPO, E-cadherin (CDH1), urokinase, XPA and XPD gene polymorphisms.

Polymorphism (location)*	Primers sequences (5'→3')	Denature (°C/s)	Annealing (°C/s)	Extension (°C/s)	Restriction enzyme (°C/min)	SNP sequence	DNA fragment size‡
IGF2 exon 9 (12)	F-CTTGACTTTGAGTCAAATTGGC;	95/20	60/20	72/20	Apa I (37°C /30 min)	A	100
	R-GCGGTACGAGCGACGTGCCAC					G	63+37
MPO-463 (15)	F-CGGTATAGGCACACAATGGTGA	95/30	60/30	72/45	Aci I (37°C /30 min)	A	289+61
	R-GCAATGGTTCAAGCGATTCTTC					G	169+120+61
E-Cadherin 3'-UTR (13)	F-CAGACAAAGACCAGGACTAT;	94/20	56/20	72/20	Pml I (37°C /30 min)	T	172 bp
	R-CACCGACCACCAAAAAGTCGAGGGAA					C	146+26 bp
Urokinase 3'-UTR (17)	F-CCGCAGTCACACCAAGGAAGAG	94/30	58/30	72/40	ApaL I (37°C /30 min)	T	210 bp
	R-GCCTGAGGGTAAAGCTATTGTCGTGCAC					C	185+25 bp
XPA A-23G (7)	F-TTAACTGCGCAGGCGCTCACTC;	94/30	60/30	72/45	Msp I (37°C /4 hours)	A	158
	R-AAAGCCCCGTGCGCCGCCCCAT					G	132+26
XPD Lys751Gln (7)	F-GCCCCGTCTGGATTATACG	94/30	57/30	72/45	Pst I (37°C /4 hours)	A	290+146
	R-CTATCATCTCTGGCCCCC					C	227+146+63

\*Modified PCR condition as previous reports. F and R indicate forward and reverse primers. ‡ Electrophoresis with 3% agarose gel.

transmembrane adhesion molecule, is one of the key molecules in epithelial cell adhesion (8). CDH1 thus plays an important role in the construction of tissues and organs (8). Reduction of cell-cell adhesion is related to a reduced expression of CDH1 (4). CDH1 is associated with invasiveness, lymph node metastasis, distant metastasis, and other poor prognostic factors (8). Another potential factor in the local regulation of tumour cell invasion is the urokinase-type plasminogen activator (uPA) system. Urokinase is a plasminogen activator that cleaves plasminogen to plasmin and hence stimulates fibrinolysis (9). The concentrations of urokinase of leiomyoma were lower than those in normal myometrium (9). The plasminogen, fibrinogen, and fibrinogen products of menstrual fluid were higher in patients with leiomyoma than those in the normal population (10).

The DNA repair system is required to maintain the genetic integrity of all tissues. Reduced DNA repair capacity might increase the susceptibility to tumorigenesis. The DNA repair pathway also plays a critical role in protecting the genome from insult by cancer-causing agents. Polymorphisms of DNA repair are important in modulating genotoxic effects and tumour susceptibility. Xeroderma pigmentosum (XP) is a group of rare autosomal recessive conditions characterized by defective DNA repair. The XP genes are involved in nucleotide excision repair of DNA (11). Several XP genes, including XPA and XPD, play important roles in determining the efficiencies of the transcription-coupled repair pathway (11).

Unlike mutations, polymorphisms are not directly linked to certain diseases. However, they are useful in studying multifactorial disorders. Most single nucleotide polymorphisms (SNPs) do not alter the transcript levels of related genes. These SNPs might be in disequilibrium with other unidentified polymorphisms, which influences the susceptibility of individual diseases cooperatively. Furthermore, DNA stability

is strongly dependent on the surrounding DNA sequence environment, complexity, first and second DNA architecture. The mutant alleles for these SNPs may serve as markers of a functional variant in a nearby gene.

Genetic studies of multifactorial diseases such as leiomyoma are difficult to undertake due to the uncertainties of polygenic traits. The identification of related genes is essential for the understanding of related pathogenesis for leiomyoma. This study aimed to evaluate whether gene polymorphisms are useful markers for predicting the susceptibility of leiomyoma and whether these SNPs are attractive markers for leiomyoma susceptibility. To the best of the Authors' knowledge, this is the first study in this field.

## Materials and Methods

Pre-menopausal Taiwanese women with surgically diagnosed leiomyoma and non-leiomyoma were recruited. All patients were divided into two groups: group 1, leiomyoma (n=158); group 2, non-leiomyoma (n=156). All individuals with leiomyoma accepted laparoscopy or laparotomy management and disease was confirmed pathologically. No individuals had been treated with exogenous hormone in the two years preceding recruitment. The studies were approved by the ethical committee and institutional review board of China Medical University Hospital. All informed consents were obtained from all participants in the study.

All women underwent peripheral blood sampling for genotype analyses. The genomic DNA was isolated from peripheral blood leukocytes by use of a genomic DNA isolation kit (Blossom, Taipei, Taiwan). A total of 50 ng genomic DNA was mixed with 20 pmol of each polymerase chain reaction (PCR) primer in a total volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 2.0 mM magnesium chloride, 0.2 mM each deoxyribonucleotide triphosphate, and 1 U DNA polymerase (Amplitag; Perkin-Elmer, Foster City, CA, USA). A total of five gene polymorphisms were surveyed, including IGF2 exon 9, MPO-

Table II. Genotype and allele frequencies of IGF2, MPO, E-cadherin, urokinase, XPA and XPD gene polymorphisms in women with and without leiomyoma.

	Leiomyoma n=158 (%)	Non-leiomyoma n=156 (%)	p-Value*
<i>IGF2</i>			
AA	60 (38)	42 (22.4)	0.007
AG	62 (39.2)	86 (53.9)	
GG	36 (22.8)	28 (23.7)	
A	182 (57.6)	154 (49.4)	0.039
G	134 (42.4)	158 (50.6)	
<i>MPO-463</i>			
AA	3 (1.9)	6 (3.8)	NS
AG	37 (23.4)	38 (24.4)	
GG	118 (74.7)	112 (71.8)	
A	43 (13.6)	50 (16)	NS
G	273 (86.4)	262 (84)	
<i>CDH1 3'-UTR</i>			
CC	44 (27.8)	33 (21.2)	0.02
CT	105 (66.5)	100 (64.1)	
TT	9 (5.7)	23 (14.7)	
C	193 (61.1)	166 (54.5)	0.046
T	123 (38.9)	146 (45.5)	
<i>Urokinase 3'-UTR</i>			
CC	1 (0.6)	2 (1.3)	NS
CT	11 (7)	7 (4.5)	
TT	146 (92.4)	147 (94.2)	
C	13 (4.1)	11 (3.5)	NS
T	303 (95.9)	301 (96.5)	
<i>XPA</i>			
AA	33 (20.9)	35 (22.4)	NS
AG	87 (55.1)	84 (53.9)	
GG	38 (24)	37 (23.7)	
A	153 (48.4)	154 (49.4)	NS
G	163 (51.6)	158 (50.6)	
<i>XPD</i>			
AA	135 (85.4)	132 (84.6)	NS
AC	23 (14.6)	24 (15.4)	
CC	0	0	
A	293 (92.7)	288 (92.3)	NS
C	23 (7.3)	24 (7.7)	

\*p-Value was calculated by  $\chi^2$  test.

463, *CDH1*, urokinase, *XPA* A-23G, and *XPD* Lys751Gln. The SNP information for the genes involved was obtained through the internet (<http://www.ncbi.nlm.nih.gov/locuslink/>).

The PCR primer sequences and conditions were designed as given in Table I. In brief, the PCR amplification was performed in a programmable thermal cycler GenAmp PCR system 2400 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). After PCR amplification, the individual gene polymorphisms were analysed by restriction digestion with restriction enzymes, including *Pml* I and *Apal* I (New England Biolabs, Inc, Beverly, MA, USA) (Table I). The subsequent reaction buffer was used according as manufacturer's instructions (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The reaction was incubated for 3 hours at 37°C. The PCR products were mixed together and 10  $\mu$ l of this solution were loaded into 3% agarose gel containing ethidium

bromide for electrophoresis. Genotypes and allelic frequencies for each gene polymorphism in both groups were compared. Correlation of these SNPs and leiomyoma was evaluated. Allelic frequencies are expressed as a percentage of the total number of alleles. The SAS system (Version 8.1; SAS Institute Inc., Cary, NC, USA) with  $\chi^2$  test was utilized for statistical analyses. A p-value of <0.05 was considered statistically significant.

## Results

No significant differences between leiomyoma and control groups were found in terms of age (30.4 $\pm$ 3.5 vs. 31.7 $\pm$ 4.4) and body mass index (21.5 $\pm$ 2.3 vs. 21.6 $\pm$ 2.1). Genotype and allelic proportions of *IGF2* exon 9 and *CDH1* polymorphisms in both groups were significantly different (Table II). Dominant percentages of *IGF2*\*A and *CDH1*\*C homozygotes presented in leiomyoma group. Proportions of *IGF2*\*A homozygote/heterozygote/G homozygote and A/G alleles in both groups were: (group 1) 38/39.2/22.8% and 57.6/42.4%; (group 2) 22.4/53.9/23.7% and 49.4/50.6%, respectively (Table II). Proportions of *CDH1*\*C homozygote/heterozygote/T homozygote and C/T alleles in both groups were: (group 1) 27.8/66.5/5.7% and 61.1/38.9%; (group 2) 21.2/64.1/14.7% and 54.5/45.5%, respectively. In contrast, genotype and allelic proportions of MPO-463 and urokinase in both groups were not significantly different (Table II). Dominant presentations of MPO\*G and urokinase\*T related genotypes or alleles presented in all individuals. Proportions of MPO\*A homozygote/heterozygote/G homozygote and A/G alleles in both groups were: (group 1) 1.9/23.4/74.7% and 13.6/86.4%; (group 2) 3.8/24.4/71.8% and 16/84%, respectively (Table II). Proportions of urokinase\*C homozygote/heterozygote/T homozygote and C/T alleles in both groups were: (group 1) 0.6/7/92.4% and 4.1/95.9%; (group 2) 1.3/4.5/94.2% and 3.5/96.5%, respectively.

Furthermore, genotype proportions of different XPA and XPD polymorphisms in both groups were not significantly different (Table II). The XPA\*A and G genotypes and alleles appear with equal distributions. Proportions of XPA\*A homozygote/heterozygote/G homozygote and A/G alleles in both groups were: (group 1) 20.9/55.1/24% and 48.4/51.6%; (group 2) 22.4/53.9/23.7% and 49.4/50.6%, respectively (Table II). All individuals in both groups had the XPD\*A-related genotypes and alleles. Proportions of XPD\*A homozygote/heterozygote/C homozygote and A/C alleles in both groups were: (group 1) 85.4/14.6/0% and 92.7/7.3%; (group 2) 84.6/15.4/0% and 92.3/7.7%, respectively.

## Discussion

Despite the high prevalence of uterine leiomyoma, or fibroids, its etiology remains obscure. Several growth factors, including IGF, epidermal GF, fibroblast GF and platelet-derived GF, are present in the uterus (12). IGF2 is involved

Table III. Association of *IGF2*, *MPO*, *E-cadherin*, *urokinase*, *XPA* and *XPD* gene polymorphisms with individual disorders.

	Association	Non-association
IGF2	Glaucoma, leiomyosarcoma, leiomyoma, ovarian tumour, endometrial cancer, cervical cancer, breast carcinoma, testicular tumour, prostate cancer, lung cancer, colorectal cancer, hepatocellular carcinoma.	Urolithiasis, prostate cancer, Beckwith-Wiedemann syndrome, Wilms' tumour.
MPO-463	Lung cancer, atherosclerosis, coronary artery disease, bladder carcinogenesis, sarcoidosis, Alzheimer's disease, hepatoblastoma.	Endometriosis, glaucoma, lung cancer, vasculitis, sarcoidosis.
E-Cadherin	Urolithiasis, prostate cancer, glaucoma, ovarian cancer, esophageal cancer, breast cancer, esophageal cancer, gastric cancer, colorectal cancer, pancreatic cancer, lung cancer, thyroid cancer, prostate carcinoma.	Ovarian cancer.
Urokinase	Urolithiasis, rheumatoid arthritis, oral cancer, endometriosis, endometrial cancer, ovarian tumour, breast cancer, atherosclerosis and coronary artery disease, hepatocellular carcinoma, colon carcinoma, leukemia, melanoma.	Bronchopulmonary dysplasia for preterm infants, osteoporosis.
XPA*-23 A/G	Lung cancer.	Oral cancer.
XPD*Lys751Gln	Melanoma, lung cancer, colorectal cancer.	Oral cancer, lung cancer.

in the growth of uterine smooth muscle tumours (5). Leiomyoma contains more *IGF2* mRNA than does myometrium, which might be related to its genesis and progression (13). *IGF2* functions as autocrine or paracrine mediator of oestrogen action in maintaining, differentiating, and enhancing tumour growth (6). *IGF* is a potent mitogen which may act as a mediator of sex steroid-induced cell growth and differentiation. The mRNA expression of *IGF2* is regulated by ovarian hormone (14). The hypoestrogenism induced by the gonadotrophin-releasing hormone analogue is also related to a decreased level of *IGF* (14). The changes in *IGF* binding to myometrium may play a role in the pathogenesis of leiomyoma (14).

*IGF2*, a 67-amino acid mitogenic peptide, plays an immunohistochemical role in leiomyoma development (14). The regulation of human *IGF2*, which is located at chromosome 11p15.5, is extremely complex. *IGF2* is involved in the growth regulation of mixed mesodermal tumours of the uterus (14). Uterine leiomyosarcoma has been shown to oversecrete *IGF2* (5). *IGF2* is responsible for the differentiation of skeletal muscle and the down-regulation of *IGF* receptor gene expression (15). Alternation or loss of imprinting of *IGF2* is related to oncogenesis through either inactivation of tumour suppressor genes or activation of growth-promoting genes (5). Biallelic expression of the gene leads to overexpression of *IGF2* peptide and increased mitogenic activity (5). The appearance of biallelic expression of *IGF2* may be an early event in tumourigenesis. The two alleles of *IGF2* that are expressed in tumour tissue may be associated with different production of *IGF2* mRNA and protein (16).

The presence of an abnormal *CDH* molecule may result in the dysfunction of the cell-cell adhesion system, further triggering cancer invasion and metastasis (17). *CDH1*, a member of the cadherin family of cell surface glycoproteins,

plays a tumour suppressor role. *CDH1*, which is found in most epithelial tissues (18), consequently plays an important role in the development of epithelial structures. Furthermore, *CDH1* is recognised as a useful tumour marker since its altered expression correlates with increased tumour aggressiveness and dedifferentiation (18). *CDH* is associated with embryogenesis, polarization, differentiation, and cell migration in inflamed tissue (17). Genetic alterations in any component of the cadherin complex may induce the loss of adhesion function.

*MPO* is a 150-kDa hemoprotein stored exclusively in the azurophilic granules of monocytes and neutrophils. *MPO* produces strong oxidants and procarcinogens, such as benzo(a)pyrene and aromatic amine intermediates, which may contribute to endothelial dysfunction and damage (19). *MPO* may act as a co-carcinogen by generating free radicals and activating aromatic amines and carcinogens (20). *MPO-463\*A* allele is related to lower *MPO* expression, as well as reduced risk of individual cancers (20). The *uPA* system is associated with cell differentiation, migration, cell-associated plasminogen activation, pericellular proteolysis, extracellular matrix degradation and intracellular signal transduction, which further influences angiogenesis, invasion and metastasis of cancer (21). Urokinase levels in endometrial cancer or endometriosis have been shown to be significantly higher than those in normal endometrium (22). *uPA* is associated with progression of clinical stage in endometrial carcinoma (22), and elevated *uPA* is correlated with myometrial invasion and unfavorable prognosis (22).

Variation in DNA repair capacity is linked to a risk of certain types of cancer. Around 40% of leiomyomas appear to have an abnormal karyotype (23). Specific chromosomal abnormalities, such as chromosome deletion, translocation, rearrangement, or trisomy, are seen in approximately 30% of

uterine leiomyomas (23). The XP-related genes may play an essential role in DNA repair, both in the global genomic repair and in the transcription-coupled repair pathways (24). Among the XP gene family, the XPA 23\*GG and XPD polymorphism have been associated with reduced repair proficiency and cancer susceptibility (11).

SNPs are the most abundant variation of the DNA sequence in the human genome. More than 1.4 million SNPs have been identified in the human genome, allowing the possibility of a genome-wide linkage disequilibrium mapping of the genes in the human population. SNPs markers have gained popularity due to the ability to analyse them quickly, accurately and inexpensively in different diseases. Such SNP analysis provides a new way for the identification of complex gene-associated diseases, such as leiomyoma. Reviewing the MEDLINE database, some studies observed the correlation or non-correlation statuses of these polymorphisms with individual diseases (Table III). However, few investigators demonstrated their correlation with leiomyoma. Vu *et al.* (5) demonstrated that the incidence of IGF2\*heterozygosity is significantly lower in patients with uterine leiomyosarcoma than in healthy individuals. They also observed that the IGF2 of myometrium and leiomyoma was always expressed monoallelically. In this study, we observed that the genotype distributions for IGF2 were significantly different between the individuals with leiomyoma and the normal population. IGF2\*A homozygosity is related to a higher risk of leiomyoma development. Heterozygosity or G homozygosity are related to lower risk of leiomyoma development, in agreement with Vu *et al.* (5).

This study also showed that the genotype distributions and allelic frequencies for CDH1 were significantly different between individuals with leiomyoma and the normal population. C homozygotes possessed a higher risk of developing leiomyoma, whereas the C/T heterozygote or the T homozygote had a lower risk. In contrast, no-association of another four polymorphisms (MPO, urokinase, XPA\*A-23G, XPD\*Lys751Gln) with leiomyoma was observed. These findings indicated these gene variations may not be good candidates as genetic markers for susceptibility to leiomyoma.

In conclusion, an association between leiomyoma and IGF2 and CDH1 polymorphisms exists. The IGF2\*A and CDH1\*C alleles were correlated with leiomyoma susceptibility, which may be associated with leiomyoma development. Therefore, the IGF2 and CDH1 polymorphisms may contribute to the pathogenesis of leiomyoma. Although the real roles of these gene variations have not yet been clarified, they should be further investigated in order to more fully understand their roles in leiomyoma development. In so doing, it will be possible to ascertain their suitability as predictive markers of leiomyoma susceptibility, and possibly

develop new early therapeutic interventions in women at high risk for leiomyoma. Furthermore, the effects of other growth factors or cadherin polymorphisms upon leiomyoma development should be investigated further. In contrast, there is no correlation between leiomyoma and the other four polymorphisms. Therefore, it can be concluded that MPO\*-463A/G, urokinase, XPA\*A-23G and XPD\*Lys751Gln polymorphisms do not appear to be related to leiomyoma susceptibility, and are thus not useful as predictive markers of leiomyoma susceptibility. However, the mapping of the nucleotide excision repair pathway and related genes may contribute to the understanding of their roles upon tumorigenesis. After the clarification of these issues, certain DNA repair gene polymorphisms may become useful markers for predicting the future development of leiomyoma as well as providing the valuable insight into the pathogenesis of leiomyoma.

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