

## RESEARCH ARTICLE

# Role of Endocrine Disrupting Chemicals in the Occurrence of Benign Uterine Leiomyomata: Special Emphasis on AhR Tissue Levels

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### Abstract

Although benign uterine leiomyomata (LMA) is the most common reproductive tumor in premenopausal women, its etiology is largely unknown. We aimed in the present study to demonstrate the potential role of environmental factors with estrogenic activity in tumor etiology by focusing on the role of aryl hydrocarbon receptor (AhR) which mediates the effects of many environmental endocrine disruptors and contributes to the loss of normal ovarian function in polluted environments. This case-control study aimed to compare the interactions between AhR and lifestyle factors in a clinical setting for the first time among 138 newly diagnosed LMA patients and 138 normal controls who lived in Tehran and Mashhad, respectively, during the last 10 years. To conduct immunohistochemical studies using appropriate monoclonal antibodies, 30 cases were selected retrospectively from 2009-2011 from the pathology departments of two university hospitals in Tehran. Although the levels of sex steroid receptors were similar in adjacent myometrium and uterine leiomyomas of all cases, AhR was significantly overexpressed ( $p=0.034$ ,  $OR=1.667$ ) in uterine LMA and this overexpression was correlated with living in Tehran [ $p=0.04$ ,  $OR=16$  (1.216-210.58)], smoking [ $p=0.04$ ,  $OR=2.085$  (1.29-3.371)], living near polycyclic aromatic hydrocarbon producing companies [ $p=0.007$ ,  $OR=2.22$  (1.256-3.926)] and eating grilled meat [ $p=0.042$ ,  $OR=1.28$  (1.92-3.842)]. Our study contributes to the understanding of the effects of EDCs on AhR levels as well as women's health and points out possible risk factors for the development and growth of uterine LMA. It seems that the development of LMA could be the result of interactions between hormonal and environmental factors.

**Keywords:** Uterine leiomyoma - leiomyomata - aryl hydrocarbon receptor (AhR) - ER - PR - AR - IHC

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### Introduction

Uterine leiomyomata (LMA) or fibroids are the most common benign female genital neoplasm among reproductive-age women (Amant, 2011). Fibroids are associated with menorrhagia, pelvic pain, spontaneous abortion, and infertility, and they account for one third of all hysterectomies in the United States (Brenda, 2007). Although uterine leiomyoma is a very common condition and its frequency can be more than 70% in premenopausal women, it has not received enough attention from researchers and the etiology of fibroids is largely unknown (Eduardo, 2001).

In recent years, exposure to exogenous estrogens, such as diethylstilbestrol in animals and increased risk of LMA

have been suggested (Hunter, 2000). In fact the cumulative exposure to exogenous estrogens from drugs, diet and persistent exposure to endocrine disrupting organic pollutants (POPs) could be important determinants in the increased risk of LMA because of their xenoestrogenic activities (Anissa, 2011). Endocrine disrupting chemicals (EDCs) are mostly synthetic compounds with estrogenic activities which are able to mimic hormones and interact with hormone transport proteins. These wide range environmental pollutants, such as polychlorinated biphenyls, dioxins, polycyclic aromatic hydrocarbons (PAHs), phthalates, bisphenol A, pesticides, alkylphenols and heavy metals (arsenic, cadmium, lead, mercury), potentially disrupt hormone metabolic pathways by mimicking the functions of endogenous hormones and, in

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some cases, completely block their functions (Balabanic, 2011; Zeliann, 2011).

The aryl hydrocarbon receptor (AhR) mediates the toxicity of EDCs with xenoestrogenic activities (Van der HE, 2009). AhR overexpression in benign (Bidgoli, 2010; 2011) and malignant breast tumors (5), (Bidgoli, 2011) and male reproductive disorders (Bidgoli, 2011) have been described recently by us. These findings were also consistent with the hypothesis that long term exposure to endogenous or exogenous estrogenic resources may down regulate the tissue levels of ER and PR and overexpress the AhR levels in mentioned tissues.

Other than environmental factors, increased risk of LMA has been reported to be related with some reproductive factors including early menarche, parity, pregnancy (Eduardo, 2001; S, 2008; Levy, 2012). Although the relationship between reproductive factors and LMA is very complex and may be altered by factors such as parity or other anthropometric characteristics (Takeda, 2008) their interaction with AhR levels is not identified yet.

On the other hand many manufacturing companies especially oil refinery in Tehran and some other capital cities of Iran release extensive amounts of PAHs with endocrine disrupting activities to the environment which are considerable comparing to the other industrialized countries (Azari, 2007) and from increasing incidence of LMA in Tehran during last decades we aimed in the present study to examine the role of PAHs in the occurrence of LMA by focusing on the differential role of AhR and sex steroid receptors in this regard. Role of reproductive and environmental factors on the tissue levels of AhR in LMA has been considered as the second part of this case control study. We tried to find more realistic etiology for this high incidence female reproductive tumor by comparing the situation between Tehran which has oil refinery center and Mashhad which is the second most populated city of Iran without oil refinery center.

## Materials and Methods

### *Population study*

A case-control study was carried out among 138 newly diagnosed LMA patients and 138 normal controls who lived in Tehran and Mashhad since last 10 years respectively. We selected cases from patients who underwent surgery from 2009-2011 in Rasool Akram and BooAli University hospitals. Cases were identified from both self-reports registration and confirmed by pathological reports. Cases who lived in cities other than Tehran and Mashhad were excluded from this study. Exclusion criteria for cases and controls were menopausal evidence, evidence of pregnancy, recent lactation and history of malignancy. Menopausal status at the time of recruitment was defined according to information on ovariectomy, hysterectomy, menstruation status (still menstruating, number of menses over the past 12 months). Women were considered postmenopausal if they had undergone a bilateral ovariectomy or if their menses had stopped 12 months or more (unless due to hysterectomy) ago. Women who were still menstruating by using

exogenous hormones and women with no information on the number of menses over the past 12 months were excluded from this study. The control group was matched with cases for age  $\pm 5$  years.

### *Exposure to EDCs*

Exact living and working addresses of cases and controls were recorded and matched with the map of oil refinery, factories generating PAHs and dioxins. A complete list of factories that release toxicants with hormone-like effects was made before starting the study. The women who lived within 4 km from the pollutant factories were considered as high risk people.

Other possible resources of exposure to EDCs including occupations during last 20 years, smoking (active versus passive), alcohol consumption, use of cosmetics and personal care products, dietary habits and using pharmacological estrogens were compared between cases and controls.

### *Reproductive variables*

Demographical variables were obtained from specific questionnaire items: 1) Delivery related factors including mother's and grandmother's age, father and grandfather's age, mother's weight and birth weight at delivery; 2) Menstruation related factors including age, weight and height at menstruation, irregular menstruation, amenorrhea and dysmenorrhea; C) Marriage related factors including marital status, age at marriage, age at first intercourse and frequency of intercourses per week.

### *Background factors*

Exact weight and height of cases and controls as well as their weight changes from menstruation to maturation, from pregnancy to breast feeding until present were recorded by pretested questionnaires. Body mass index (BMI) was calculated and compared between cases and controls.

### *Nutritional factors*

Nutritional habits were recorded to assess the daily consumption of PAHs through smoked, grilled and baked foods (especially the burned parts) by validated questionnaires (Shu, 2003).

### *Other lifestyle factors*

Personal history of endocrine disorders, background disorders, pattern of physical activity, occupation, smoking (active versus passive), alcohol consumption, history of drug regimens, radiation exposure, weight gain after age 18 were recorded by pretested specific questionnaire.

### *Immunohistochemical studies*

As previously described (Bidgoli, 2010), dewaxed and rehydrated tissue sections were subjected to antigen retrieval using microwave oven and boiling citrate buffer (pH=6.0). Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating sections by 0.3% hydrogen peroxide in methanol for 30 min and 3% BSA for 60 min, respectively. Sections were then incubated 30 min at Room Temperature with AhR mouse monoclonal

antibody (clone PRT9, abcam), ER alpha (Clone 105, Dakocytomation), PgR (Clone 636, Dakocytomation), AR (clone 441, abcam) that recognize the nuclear and cytoplasmic expression of human proteins in uterine tissues. The results were visualized using the envision system (Dakocytomation) based on the manufacturer's instruction with necessary modifications. Sections were also counterstained with Meyer's haematoxyline. In each series, a section in which incubation with the primary antibody was omitted used as negative control. The ideal staining conditions were established in our preliminary experiments. Staining was considered negative only after careful examination of the entire tissue section.

#### Scoring methods

Immunohistochemistry is an indispensable research and diagnostic tool used to assess the presence or absence of molecular tumor markers on paraffin-embedded tissue. Tumor positivity for a given marker is frequently evaluated using predetermined cutoffs such as 5% (<5% tumor cells staining=negative, >5%=positive). The employment of categorical scoring systems is motivated by the ease of interpretation of positive tissue by two independent pathologists and is further supported by substantial inter observer agreement. For all cells, staining intensity and pattern were evaluated using the semiquantitative scale of 0-2+. Each cell type in surgical samples was then classified into five categories based on the expression patterns of markers. In cases in which the observers disagreed in final score, the immunohistochemical scoring was repeated to agree on same scoring by both observers. Adding the two scores gives a maximum score of 5. All cells in each tissue section were considered by 100x, 250x and 400x magnifications.

#### Ethical considerations

Due to ethical considerations, we selected our paraffinized tissues from archives of department of Pathologies of both university hospitals.

#### Statistical analyses

Values were expressed as percent per population or as the mean±standard deviation (SD). To assess the association between expressions of markers and clinicopathological data nonparametric chi-square test was used. Relative risks and Odds ratios were calculated by Cochran's and Mantel Haenszel statistics using SPSS 17. Probability values of <0.05 and Odds ratios >1 were considered significant.

## Results

### Expression of sex steroid receptors in LMA

**A: Estrogen Receptor alpha (ER alpha):** Either myometrium or leiomyoma were reactive to ER alpha and showed strong ER nuclear staining (Figure 1). The level of ER alpha was not significantly different between adjacent myometriums and myometriums of normal controls (p=0.48).

**B: Progesterone Receptor (PgR):** Except one case which expressed the PgR slightly lower (2+) the rest of samples showed strong nuclear expression of PgR. No significant difference was recorded between PgR levels in myometrium and leiomyoma that means similar levels of PgR expression were detected in adjacent normal tissues. The level of PgR was not significantly different between adjacent myometriums and myometriums of normal controls (p=0.57).

**C: Androgen Receptors (AR):** Neither myometrium nor leiomyoma was reactive to AR monoclonal antibodies. AR was detectable in less than 10 percent of normal myometrium of control group too.

### AhR expression in LMA

At first view the level of AhR was not significantly different between adjacent myometriums and myometriums of normal controls (p=0.937) and both groups showed weak (-/+) AhR expression. Interestingly AhR was overexpressed in 37% of LMA which reported by 2+ scores. The adjacent myometrium showed negative to mild staining (-/+) in comparison to LMA tissues (Figure 2). In general strong AhR expression were found only in LMA (p=0.034, OR=1.667).

**Association between AhR expression and reproductive factors:** Although some reproductive factors including lower age at menarche ≤13 year (p=0.048, OR=1.666, 95%CI 1.023-2.713), Irregular menstruation (p=0.004, OR=2.095, 95%CI 1.293-3.393) and dysmenorrhea (p=<0.001, OR=3.194, 95%CI 1.883-5.416) in women with

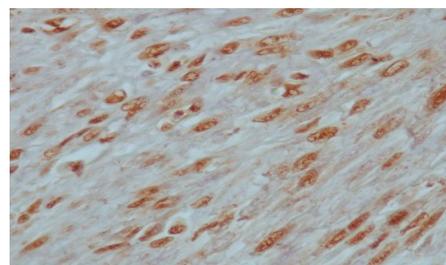


Figure 1. Strong Nuclear Expression (2+) of ER Alpha in UL

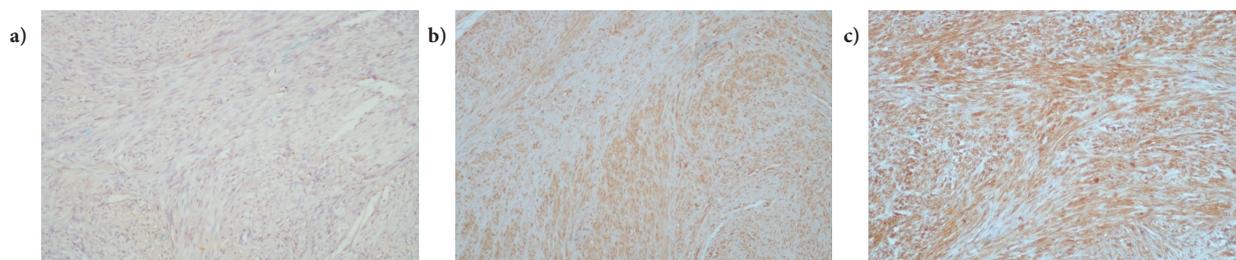


Figure 2. Expression Patterns of AhR in Uterine Leiomyoma. 1a: Negative staining 1b: Mild Staining 1c: Strong Staining

LMA was more prevalent in comparison to control group and although the history of pregnancy and lactation showed a strong protective role on the incidence of LMA ( $P < 0.001$ ,  $OR = 0.051$ ,  $95\%CI 0.007-0.39$ ), no association was identified between AhR levels and reproductive background.

An inverse association between long term (>5 years) OCP intake and decreased risk of LMA was recorded in present study. The prevalence of OCP consumption was 47.45% in cases and 50.72% in controls ( $p = 0.63$ ,  $OR = 0.877$ ,  $95\%CI 0.546-1.408$ ) but the prevalence of long term (>5 years) OCP intake was 33.33% in cases and 57.38% in controls ( $p = 0.011$ ,  $OR = 2.692$ ,  $95\%CI 1.299-5.553$ ). Level of AhR expression was not correlated with the patterns of OCP intake.

**Association between AhR expression and background factors:** 1) Familial history: Incidence of LMA in first degree family of cases (17/39%) was significantly higher than control group (5.07%), ( $p = 0.02$ ,  $OR = 3.94$ ,  $95\%CI 1.637-9.485$ ). This background factor was not correlated with AhR levels. 2) Anthropometric factors: These factors including weight, height, BMI and BMI changes after menstruation and wrist circumference were not associated with increased risk of LMA or AhR levels. 3) Economical Status and living addressee: Increased risk of LMA was determined in women who had lower economical status and lived near PAH producing manufacturing companies. That means 82.6% of LMA cases lived in polluted areas because of economical restrictions whereas the same level was 68.15% in control group ( $p = 0.007$ ,  $OR = 2.22$ ,  $95\%CI 1.256-3.926$ ). 4) City of residence: Tissue levels of AhR were compared between residents of Tehran ( $n = 50$ ) and Mashhad as the second big city of Iran. Out of 18 strong AhR positive cases (2+), 16 cases (94.12%) were residents of Tehran ( $p = 0.04$ ,  $OR = 16$ ,  $95\%CI 1.216-210.58$ ) whereas half of AhR negatives were Tehran residents and the rest were residents of Mashhad (Figure 3). 5) Cigarette Smoking : Exposure to cigarette smoke increased the

**Table 1. Risk Factors of Uterine LMA in Iranian Women: Association with AhR Expression**

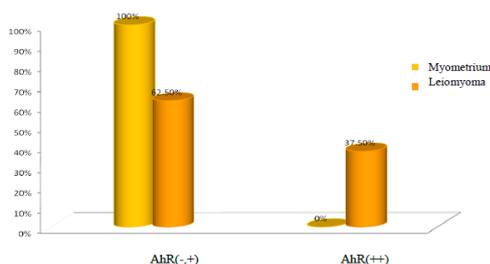
Importance of Risk Factors	OR (95%CI)	P-value	Association with AhR
<b>Reproductive factors:</b>			
Early menarche ( $\leq 13$ year)	1.66 (1.883-5.416)	0.048	NO
Irregular menstruation	2.095 (1.293-3.393)	0.004	NO
History of pregnancy and lactation			
	0.051 (0.007-0.39)	0.001	NO
Short term OCP consumption (>5yrs)			
	2.692 (1.299-5.553)	0.011	NO
<b>Background factors:</b>			
Familial history	3.94 (1.637-9.485)	0.02	NO
<b>Environmental factors:</b>			
Living near PAH producing manufacturing companies			
	2.22 (1.256-3.926)	0.007	Yes
City of residence	16 (1.216-210.58)	0.04	Yes
Smoking	2.085 (1.29-3.371)	0.004	NO
<b>Nutritional factors:</b>			
Eating barbecued and grilled meats (>3 times/week)			
	1.28 (1.92-3.842)	0.042	Yes

risk of LMA that means 56.5% of cases were exposed to the cigarette smoke whereas the same level was 38.4% in controls ( $p = 0.004$ ,  $OR = 2.085$ ,  $95\%CI 1.29-3.371$ ). The tissue levels of AhR was not affected by exposing to cigarette smoke. 6) Daily intake of PAHs by food: Although no direct association between increased risk of LMA and eating barbecued and grilled foods was detected, AhR levels were higher in the group of patients who used grilled meat routinely. In fact 77.78% of AhR positives (2+) were cases who had grilled meat (kebab) more than 3 times per week in their food regimen ( $p = 0.042$ ).

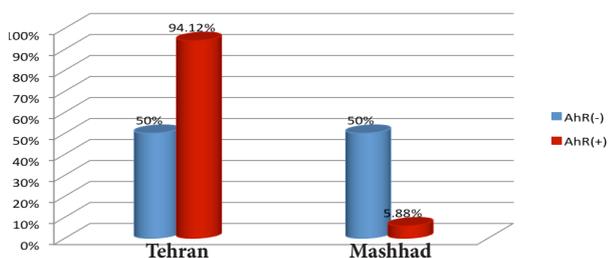
**Discussion**

Endogenous activation of ovarian hormones by reproductive factors have been suggested as the major risk factor of LMA in some population (Eduardo, 2001) but the remarkable frequency of uterine LMA in the human population calls into the question on the potential role of environmental factors with estrogenic activities in tumor etiology (Levy, 2012). The role of endocrine disruptors as the main source of exposure to exogenous estrogens has been focused in present study on the basis of tissue levels of Aryl hydrocarbon Receptor (AhR). AhR mediates the effects of many xenoestrogens and endocrine disruptors and contributes to the loss of normal ovarian function in polluted environments (Sugiura-Ogasawara, 2005; Valdez, 2009). We have detected its roles on the incidence of LMA in this case control study which was conducted in the most polluted and populated city of Iran, Tehran comparing with Mashhad as the second most populated city (not polluted) of Iran. We considered the existence of refinery center with 220,000 bbl/d installed capacity and extensive diesel exhaust in Tehran as the major source of difference between these two mentioned cities. Other than citizenship factors we looked for exposure to many other resources of exposure to endocrine disruptors by recording their food habits and lifestyle factors which may induce the AhR levels in target tissues (Zeliann, 2011).

This study has demonstrated wide expression of ER alpha and PR in nearly 100% of uterine LMA and low



**Figure 3. Comparison of AhR Levels between Myometrium and Leiomyoma**



**Figure 4. Comparison of AhR Levels between Tehran and Mashhad Residents**

level of AR expression in the uterine tissues which were in accordance to previous studies (Kawaguchi, 1991; Leitao, 2004). Although the level of sex steroid receptors were similar in adjacent myometriums and uterine leiomyomas of all cases, AhR was strongly stained and significantly overexpressed ( $p=0.034$ ,  $OR=1.667$ ) in uterine LMA.

Our study demonstrates that AhR is widely expressed in human reproductive tissues as we previously described it in benign and malignant breast tumors (Bidgoli, 2010; 2011a; 2011b). Despite breast tumors this transcription factor shows a similar pattern of distribution in uterine tissues without any antagonistic relationships with hormone receptors but AhR was overexpressed in 37% of LMA and this overexpression was correlated with environmental factors clearly. AhR overexpression was significantly affected by few numbers of environmental factors especially the city of residence as the most important risk factor of AhR expression (Figure 3). Other than living in Tehran, AhR was overexpressed in women who lived near PAH producing companies, who were active or passive smokers and who ate barbecued and grilled meats (>3 times/week). Other studies have reported high levels of AhR mRNA expression in the human placenta, endometrium and endometriosis and leiomyomas (Khorram, 2002) but to our knowledge, this is the first report on AhR protein expression and its association with environmental factors in uterine LMA. In fact this research showed how the higher levels of AhR could be used as a possible biomarker of exposure to PAHs by food and air pollution. It seems that overexpression of AhR in LMA is in all important enough to suggest AhR as a possible potential reason for this very prevalent reproductive disease in a very polluted environment like Tehran.

According to the latest findings up to 40% of uterine LMA showed some chromosomal abnormalities and the most common ones are aberration of chromosomes 6, 7, 12 and 14. It is suggested also that LMA have been linked to mutations of fumarate hydratase (FH) gene. Germline mutations in FH gene cause autosomal dominant syndromes MCUL1 (multiple cutaneous and uterine leiomyomata) and HLRCC (hereditary leiomyomatosis and renal cell cancer), characterized by multiple uterine and cutaneous leiomyomata and renal cancer (Kubínová, 2012). Association between incidence of LMA in first degree families and increased risk of this abnormality was detected in this study which was not associated with AhR levels but it seems necessary to find the possible association between AhR levels and chromosomal abnormalities in the next studies.

Uterine LMA tumors is thought to depend on ovarian hormones and it is known that these tumors are hyper-responsive to estrogen and contains increased levels of estrogen receptors (Wise, 2004) as we showed in the first part of our study. On the basis of this strong hypothesis we tried to find the reproductive etiology for uterine LMA in present case control study for Iranian population. As the other epidemiological studies in white populations described (Wise, 2004) we have demonstrated an inverse association with age at menarche, parity, pregnancy and lactation and increased risk of LMA. Long term intake (>5

years) of oral contraceptive decreased the risk of tumors which is inconsistent with previous works (Samadi, 1996; Marshall, 1998) but tend toward an inverse association, particularly for preparations with a higher dose of progestin in Iranian OCPs.

With the realization of the presence of many ubiquitous xenoestrogens in our environment and food and the high incidence of uterine leiomyomas in women in Iran, the potential influence of food regimen in the pathogenesis of these tumors has been called into question in this study. Previous data suggest a reduced risk of LMA among women with a greater dietary intake of fruit and preformed vitamin A (Wise, 2011) but this result was not repeated in this study.

In conclusion, in summary, we believe that the data presented here will shed some light on a possible association between exposure to EDCs and PAHs by living in contaminated cities and polluted areas and increased risk of Uterine LMA. It seems that these factors should be strongly considered as a possible risk factor for LMA in women. In conclusion, we report for the first time the environmental risk factors of Uterine LMA and their contribution to the extent of AhR expression. Our study contributes to the understanding of the effects of EDCs on AhR levels as well as women's health and point out possible risk factors for the development and growth of uterine LMA. It seems that incidence of LMA could be the result of interactions between hormonal and environmental factors. Further studies are necessary to find the possible interactions between AhR expression and other tumor related proteins as well as the molecular mechanism of AhR in uterine LMA progression.

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