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YAP expression in neoplastic and non-neoplastic breast tissue of women chronically exposed to Arsenic

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Abstract

The Hippo pathway regulates cell proliferation and apoptosis and it has been noted that loss of critical components of this pathway can lead to uncontrolled cell growth. Yap protein is an important component of this HIPPO pathway because YAP is the nuclear effector of the Hippo tumor suppressor pathway and it is crucial for the response to oxidative stress induced by cellular process and for different xenobiotics including arsenic (As). It has been proposed that YAP dysregulation can contribute to a malignant cellular phenotype acting as both a tumor suppressor and an oncogene.

The aim of the study was to assess and compare the expression of YAP in neoplastic and non-neoplastic breast tissue of women chronically exposed to As through drinking water.

YAP expression was assessed by immunohistochemistry in 120 breast biopsies from women with breast cancer and from women with other non-neoplastic breast pathologies. Arsenic concentration was quantified in urine and toenails.

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Statement of author contributions: RRV and GLOG conceived and designed the study. GMR, TGC, EPS, MDM and MDG, recruited study participants and collected the data. GMR and TGC processed the samples. RRV, GMR, TGC, RCL and AJG drafted the initial manuscript, and all authors revised the manuscript for critical intellectual content and read and approved the final version for submission.

The results disclosed a lower percentage of YAP expression in the nucleus and in the cytoplasm in cases when compared to the registered in controls. High As levels decreases mainly YAP expression at the nucleus. YAP high intensity staining decreases the risk for breast cancer.

The overall data suggest that YAP may acts as a tumor suppressor protein and that As is able to reduce the YAP translocation from the cytoplasm to the nucleus which can induce an environment favorable for inhibition of apoptosis and promoting cellular proliferation by increasing the genomic instability of cells.

Keywords

Yes Associated Protein; YAP; arsenic; breast cancer

Introduction

The process of carcinogenesis is so complex, and it is believed that multiple mechanisms contribute to the development of cancer with disruption of the equilibrium between cellular proliferation and apoptosis [Yu et al., 2013].

The Hippo tumor suppressor pathway coordinates cellular proliferation, cell death and cellular differentiation [Gomez et al., 2014]. The dysregulation of this pathway has been linked for human cancer since 2002 [Halder and Johnson, 2011], and it has been proposed that this dysregulation could due to the loss of critical components of the Hippo pathway, which can lead to uncontrolled cell growth [Olson, 2005].

Among the main components of Hippo pathway is the mammalian transcriptional coactivator Yes-associated protein (YAP). This protein is considered as a nuclear effector of HIPPO pathway [Dong et al., 2007; Pan D, 2007]. YAP was discovered in 1994 and since then, knowledge of its function has been increased and one of these functions is its regulatory role in signal transduction and gene transcription in normal cells [Chen et al., 2010]. YAP as transcriptional coactivator controls the cellular responses through interaction with TEA domain family of growth-promoting transcription factors in the nucleus, while its transcriptional functions are inhibited by phosphorylation-dependent translocation to the cytosol [Vlug et al., 2013], which results in the reduction of transcription from its target genes.

Normally, YAP is expressed in the cytoplasm and in the nucleus, however, the expression of YAP in the nucleus of normal tissue likely represents the normal physiologic function of YAP [Ideker et al., 2001], where it plays an important role in the cellular homeostasis [Dong et al., 2007; Pan D, 2007]. Abnormal YAP expression have been linked to different kinds of cancers (liver, stomach, ovary, prostate, colon, esophagus, breast and in glioblastomas) [Su et al., 2012; Wang et al., 2010]; however, little is known about the regulation mechanism of YAP in breast tissue and there are contrasting reports about its function in this specific kind of cancer. Several dissimilar studies have demonstrated that YAP possesses oncogenic or tumor suppressor functions. Indeed, some of them have been demonstrated that YAP acts as a tumor suppressor in breast cancer (BC), showing decreased level of YAP expression in

human breast cancer tissue relative to normal breast tissue [Tufail et al., 2012], increased cell migration and invasiveness of YAP-downregulated breast cancer cells *in vitro*, and increased tumor growth in the YAP-knockout mouse *in vivo* [Yuan et al., 2008]. On the contrary, other researchers have stated that YAP acts as an oncogene in breast cancer, demonstrating a cellular overexpression of YAP [Zhi et al., 2012]. These previous studies have reported different frequencies of cellular YAP expression, one of them reported a positive staining in 75% of the breast tissue samples assessed [Wang et al., 2012]; meanwhile, another reported a frequency of 45% [Tufail et al., 2012].

Additionally to the YAP functions commented previously, YAP is crucial for the response to oxidative stress induced by cellular process and for different xenobiotics as well as for resistance to cytotoxic agents and heavy metals [Toone and Jones, 1999] including arsenic (As). It has been reported that As induces the Hippo signaling pathway and contributes to activate it aberrantly, which in turn, could contribute to the pathogenesis of epithelial neoplasm. [Li et al., 2013]. Arsenic exposure has been recognized as a contributor to the etiology of BC. Higher As levels have been detected in BC patients when compared with the found in controls [Benderli et al., 2011; Joo et al., 2009]. Recently, Muszynska et al. [2012], reported in Polish women with *BRCA1* mutation, a significantly higher risk for breast cancer based on higher serum arsenic levels.

Then, some works have been linked abnormal YAP expression to BC, and others have associated breast cancer with arsenic levels; however, no studies have associated the expression of YAP in normal breast tissue and in breast neoplastic cells with arsenic concentrations and for this reason the present study was carried out.

Material and Methods

Study population

A cross sectional study was implemented and participants were recruited from Department of Gynecologic-Oncology, Mexican Institute of Social Security, Torreon, Coahuila, Mexico. Eligible patients included women >18 years old and who had at least 10 years of residence in the Comarca Laguna, Mexico. We excluded all patients with other type of cancer or who had received radiotherapy or chemotherapy prior to the study. A total of 120 women were included. The cases were 76 newly diagnosed women with a first diagnosis of BC identified by biopsy. The controls were 44 women with biopsies negative for malignancy who came from the same hospitals and geographical area. Written informed consent was obtained from each participant. The study protocol was approved by the Ethics Committee of the School of Medicine at Torreon, University of Coahuila, Mexico.

Questionnaire application

Information was collected through in-person interviews and included sociodemographic variables (education, socioeconomic status (low, medium and high), residential history (years), reproductive history (age of menarche, age at first pregnancy, number of pregnancies, parity, months of lactation, history of hormonal contraception or replacement therapies (years), age of menopause, lifestyle factors (smoking, alcohol intake and exercise),

a detailed family history of cancer (any family member), occupational history, history of As exposure and diet.

Immunochemistry

Paraffin-embedded tissue blocks were sectioned (4 μm) into silanized slides. Slides were deparaffinized in xylene and rehydrated in a graded alcohol series. Antigen retrieval was achieved by Target Retrieval Solution Envision Flex at 98°C for 20 minutes in Pt link system (PT10027, California, USA). Endogenous peroxidase was removed with 3% peroxidase-blocking reagent (Dako®) for 10 minutes at room temperature. Then slides were incubated with rabbit polyclonal antibody against human YAP (1:150 Abcam®) diluted with antibody diluent from Dako® for 1 hour in a humidity chamber at 4 °C followed by TBSt (Tris saline buffer and tween 20) wash. Sections were incubated with horseradish peroxidase (HRP) from Dako® for 20 minutes. Detection was performed using diaminobenzidine chromogen diluted in substrate buffer for 5 minutes. Slides were contrasted with hematoxylin, dehydrated and mounted. A negative control was incubated with antibody diluent without YAP antibody and a known positive control was included in each run. All non-tumor and tumor slides were reviewed by two blinded reference pathologists.

Grading for YAP immunoreactivity

The final immuno-histo-chemistry staining results are expressed as the product of the proportion of stained cells and the immunostaining intensity. Stained cell proportions were scored as follows: no staining or negative, low (less than 30% of all cells stained), and high (greater than or equal to 30% of all cells stained) [Steinhardt et al., 2008]. The immunostaining intensity was scored as follows: negative, low and high.

As measurement in urine

Individual exposure was assessed based on urinary concentration of the total arsenic level. A first morning void urine sample was collected in sterile 120-mL screw-topped polypropylene containers.

Urine samples were analyzed using the methodology described by the U.S. Center for Disease Control [CDC, 2004] at the Arizona Laboratory for Emerging Contaminants, University of Arizona, Tucson, Arizona, U.S.A. Briefly, arsenic species in urine (As^{V}, As^{III}, monomethylarsonic acid (MMA^{V}), dimethylarsinic acid, (DMA^{V}) and arsenobetaine) were separated by HPLC and analyzed by ICP-MS. Arsenic concentrations in urine were analyzed by inductively coupled plasma mass spectrometry utilizing Standard Reference Water, SMR 1640 (NIST, Gaithersburg, MD, USA) and freeze-dried Urine Reference Material for trace elements (Clincheck-control; RECIPE Chemicals instruments GmbH, Munich, Germany) for urine as quality control. Urinary As concentrations were adjusted by urine creatinine levels. Additional exposure to other arsenic compounds, which usually is attributable to consumption of rice, seafood such as bivalves and seaweeds, was considered minimal because such seafood is essentially little eaten in this area. Arsenic metabolism efficiency was calculated using the following formulas proposed by Del Razo [Del Razo et al., 1997]: first methylation = $MMA^{V}/(As^{V}+As^{III})$; second methylation = DMA^{V}/MMA^{V}.

Statistics

Independent and dependent variables were described according to their frequency and distribution measurements (arithmetic mean and standard deviation). The F test was used when the variable was divided into more than two categories, and the Student's t test or the Mann Whitney test was used to compare different YAP expression and levels of arsenic when dichotomous variable categories were analyzed. This method permitted us to establish statistical differences among groups for each dependent variable. Linear regression models were used to assess crude or independent associations between the different YAP expressions with arsenic urine concentrations, as well as, ORs was calculated. In all multivariable models, we included those statistically significant variables ($P < 0.05$) identified in the bivariate model (age, YAP status, schooling and type of drinking water). All analyses were performed using the statistical software STATA 11.0 (Stata Corp., College Station, TX).

Results

A total of 120 women were included in the study, their anthropometric, sociodemographic characteristics and lifestyle are reported in Table I. The mean age and schooling were significantly higher in controls than in cases; no other differences were found between the studies groups (Table I).

Nuclear and cytoplasmic YAP expression was assessed in 120 patients with normal breast tissue biopsies (n=44) and in neoplastic tissues (n=76). Seventy nine percent of all samples analyzed were positive for YAP expression; however, the frequency of negative YAP expression was significantly higher in cases (26.3%) than in controls (11.3%) (p=0.05) (Table II).

The frequency of Yap expression in normal breast tissue and carcinoma samples was detected mainly in the cytoplasm and thereafter in the nucleus; however, in the control group the percentage of YAP positive staining in the cytoplasm and in the nucleus was higher than in the cases (Table II).

YAP expression intensity was scored as negative, low or high. When the YAP expression intensity was evaluated in the cytoplasm, a statistically significant difference (P=0.02) were found between controls vs cases. The high YAP expression intensity in cytoplasm was greater in controls than the observed in cases; meanwhile, the negative YAP expression intensity was more frequent observed among cases (Table II). When the YAP expression intensity was recorded in the nucleus between cases and controls, no statistical significant differences were found; however, the negative YAP expression intensity was more frequently observed in cases (Table II).

When the risk for developing breast cancer according to the positive YAP staining or YAP expression intensity, the results disclosed that only the high expression intensity at the cytoplasm decreased significantly the risk for cancer (Table III).

When the urinary and toenail arsenic concentrations were compared between the studied groups, the %MMA was significantly higher in the control subjects, whereas, the second methylation's values were greater in the cases ($P<0.05$). No other differences in total As or As species were found between groups (Table IV). When the studied population was divided according with its total arsenic urinary levels (<50 and >50 μg/L), the positive staining at the nucleus was 2.8 times higher in those with the low concentrations, and however, this difference was no statistically significant (Table V). An increased risk for the development of cancer was only found with the second methylation (adjusted OR=1.25; 95% CI 1.008 – 1.55; P=0.042) (Table VI).

Discussion

Recent studies have shown that an abnormal YAP expression is associated with breast cancer, and according with its expression level, YAP has been proposed to act as both a tumor suppressor (decreased YAP expression) or an oncogene (increased YAP expression). Our results suggest that YAP has acts as a tumor suppressor because the breast cancer tissue assessed reveals decreased YAP expression compared with normal breast tissue and these results are in agreement with recent results published in other studies [Jaramillo-Rodrıguez R. et al., 2014; Tufail et al., 2012; Wang et al., 2012; Yuan et al., 2008]. Normally, YAP is distributed in the cytoplasm and in the nucleus, however, the expression of YAP in the nucleus of normal tissue likely represents the normal physiologic function of YAP [Ideker et al., 2001], where it plays an important role in the cellular homeostasis [Dong et al., 2007; Pan D, 2007]. Our data support the mentioned previously because when the risk for breast cancer was evaluated, the expression of YAP in cytoplasm and nucleus, as well as, the high expression intensity, decreased significantly the risk for cancer; therefore, its expression at these cellular sites could be used as a biomarker for breast cancer as suggested by Sang et al. [2014].

It has been proposed that the phosphorylation of YAP by specific kinases regulates its cellular and nuclear distribution and that the sequestration of phosphorylated YAP in cytoplasm results in the reduction of transcription from its target genes [Strano et al., 2001; Tufail et al., 2012]. Basu et al. [2003] showed in their study, that effectively when YAP is phosphorylated by Lats kinase, phosphorylated YAP is sequestered from the nucleus and transcription activities of target genes were reduced. In our work, we found a decreased YAP expression in the nucleus and in the cytoplasm in both studied groups; however, this decrement was higher in cases (65% and 26%, respectively) than in the controls (52% and 11%, respectively). Then, the diminution of YAP expression at the nucleus observed in our study and in other works could to be due to a sequestration of YAP in cytoplasm, thereby reducing its ability to function as nuclear effector of the Hippo tumor suppressor pathway, which in turn will propitiate an environment favorable for inhibition of apoptosis and cellular proliferation by increasing the genetic instability of premalignant cells which lead to an uncontrolled breast cell proliferation and cancer.

Other important functions described for YAP are that related with the cellular redox environment and with the genomic homeostasis. YAP plays an important role for the response to oxidative stress induced by cellular process and for different xenobiotics as well

as for resistance to cytotoxic agents and heavy metals [Toone and Jones, 1999]. Among these last compounds, we found As, which is classified as class I human carcinogens by the International Agency for Research on Cancer [IARC, 2012.]. Arsenic-induced oxidative stress and the exposure to this metalloid have been recognized as a contributor to the etiology of breast cancer. It has been reported that BC patients have significantly higher arsenic levels compared with controls [Benderli et al., 2011; Joo et al., 2009]. Recently, Lopez-Carrillo et al. [2014] found a negative association in patients with breast cancer with the major component of total As in urine (DMA). In accordance with the results reported by Lopez-Carrillo et al. [2014] we found that the second methylation levels (DMA^V/MMA^V) increase the risk for breast cancer, but we did not found significate differences in total arsenic urinary levels between cases and controls as was reported previously.

Among the different toxic mechanisms by which As propitiates the development of cancer, it is that this metalloid causes oxidative and nitrosative stress through the production of reactive oxygen species and reactive nitrogen species [Thomas-Schoemann et al., 2012]. Experimental studies have reported that when cells are exposed to oxidants and under metalloid exposure, Yap1p transiently accumulates in the nucleus [Delaunay et al., 2000; Kuge et al., 1997; Kuge et al., 2001; Yan et al., 1998] and activates transcription of genes coding for proteins that maintain a favorable cellular redox balance (*GSH1*, *TRX2*, *TRR1*, *GLR1*, and *GRE2*), as well as, for enzymes involved in detoxification of reactive oxygen species [Gasch et al., 2000; Lee et al., 1999; Toone and Jones, 1999]. The induction of the genes responsible of the synthesis of these detoxification enzymes is largely absent in *yap1*_cells. Therefore, the metalloid sensitivity of *yap1*_cells can be attributed to, at least in part, a lack of transcriptional activation of oxidative stress defense genes [Toone and Jones, 1999], which could contributes to the development of cancer.

With respect to the relation between genomic homeostasis and As, it is also known that As activate, induce or alter the phosphorylation-dependent signaling pathways including the Hippo signaling pathway and which is involved in the control of cell proliferation, adhesion and migration and if it is abnormally activated, induced or altered, it contributes to the pathogenesis of neoplasms [Alp et al., 2010; Li et al., 2013]. It has been reported that As treatment enhances phosphorylation-dependent activation of LATS1 kinase and other Hippo signaling regulatory proteins Sav1 and MOB1, as well as, that Phospho-LATS kinase catalyze the inactivation of a transcriptional co-activator, Yap [Li et al., 2013]. In our study, when the studied population was divided according with their As urinary levels, those subjects with >50 μg/L had 2.8 times less YAP expression at the nucleus.

Then and according with the previously mentioned, As exposure disrupt the Hippo signaling pathway through phosphorylation which decreases YAP expression at the nucleus as was documented in our study which in turn modify the cellular redox environment and the genomic homeostasis which contributes to the pathogenesis of cancer.

The overall data suggest that YAP may acts as a tumor suppressor protein and that As is able to reduce the YAP translocation from the cytoplasm to the nucleus which can induce an environment favorable for inhibition of apoptosis and promoting cellular proliferation by increasing the genetic instability of cells.

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Table I

Anthropometric, socio-demographic characteristics and lifestyle factors of participants. Results shown as arithmetic mean and standard deviation (percent).

	Cases (n=76)	Controls (n=44)	*P*- value
Age (years)	43.17 ± 10.4	52.90 ± 13.6	0.00
Body mass index	29.62 ± 5.7	28.39 ± 6.8	0.40
Schooling (years)	8.51 ± 4.9	11.0 ± 4.6	0.03
Age at menarche (years)	12.75 ± 1.2	12.66 ± 1.7	0.78
First childbirth age (years)	21.24 ± 4.7	20.65 ± 2.8	0.57
Number of **c**hildbirth	3.90 ± 2.6	3.33 ± 1.5	0.33
Breastfeeding (months)	7.84 ± 9.7	6.69 ± 10.1	0.62
Age at menopause (years)	45.45 ± 1.0	44 ± 8.2	0.51
Time living in Comarca Lagunera (years)	44.53 ± 16.6	37.79 ± 16.48	0.89
Type of water used for drink n (%)			
Purified	36 (47.3)	33 (75.0)	0.44
Tap water	36 (47.3)	9 (20.4)	
Both	4 (5.2)	2 (4.5)	
Type of water used for cook n (%)			
Purified	16 (21.05)	9 (20.45)	0.09
Tap water	56 (73.68)	26 (59.09)	
Both	4 (5.26)	9 (20.45)	

Chi^2 or t test. P <0.05.

Table II

YAP expression in breast tissue. Results are shown as frequency (percent).

	Cases (n=76)	Controls (n=44)	*P* value
YAP location n (%)			
Cytoplasmic			
Negative	20 (26.3)	5 (11.4)	0.05
Positive	56 (73.7)	39 (88.6)	
Nuclear			
Negative	50 (65.8)	23 (52.3)	0.10
Positive	26 (34.2)	21 (47.7)	
YAP intensity n (%)			
Citoplasmic YAP			
Negative	20 (26.32)	5 (11.36)	0.02
Low	26 (34.21)	11 (25.00)	
High	30 (39.47)	28 (63.64)	
Nuclear YAP			
Negative	50 (65.79)	23 (52.27)	0.32
Low	10 (13.16)	9 (20.45)	
High	16 (21.05)	12 (27.27)	

Chi^2 test $P<0.05$

Table III

Odds ratio for breast cancer and cellular YAP expression.

	OR	95% IC	*P* value
YAP location n (%)			
Cytoplasmic			
No staining			
Positive	0.358	0.124 – 1.038	0.06
Nuclear			
No staining			
Positive	0.569	0.266 – 1.215	0.15
YAP intensity n (%)			
Citoplasmic YAP			
Negative			
Low	1.56	0.67 – 3.58	0.29
High	0.37	0.17 – 0.80	0.01
Nuclear YAP			
Negative			
Low	0.58	(0.21 -1.58)	0.29
High	0.71	(0.30 – 1.68)	0.43

Table IV

Urinary and toenail arsenic concentrations by cases and controls. Results shown as arithmetic mean and standard deviation

	Cases (n=76)	Controls (n=44)	*P* value
Urinary arsenic metabolites (μg/L)			
As^{III}	2.85 ± 0.48	2.86 ± 0.45	0.119
As^{V}	3.26 ± 0.59	2.49 ± 0.73	0.519
MMA^{V}	5.31 ± 1.23	6.22 ± 1.13	0.112
DMA^{V}	34.77 ± 5.76	33.97 ± 5.28	0.348
Total As	46.21 ± 7.49	45.55 ± 6.80	0.395
%iAs	15.77± 2.02	14.11 ± 2.08	0.591
%MMA	10.50 ± 0.52	13.89 ± 1.40	0.020
%DMA	73.71 ± 2.00	71.99 ± 2.49	0.181
Methylation profile (μg/L)			
Primary methylation	1.10 ± 0.10	1.64 ± 0.41	0.211
Secondary methylation	9.14 ± 0.95	6.22 ± 0.57	0.046
Toenails arsenic (μg/g)	0.70 ± 0.09	0.98 ± 0.26	0.411

Mann-Whitney test. $P<0.05$

Table V

YAP location and intensity according with Arsenic urinary levels. Results are shown as frequency (percentage).

	As < 50 μg/L (n=75)	As > 50 μg/L (n=45)	*P* value
YAP location n (%)			
Cytoplasmic			
Negative	30 (40.00)	19 (42.22)	0.92
Positive	45 (60.00)	26 (57.78)	
Nuclear			
Negative	52 (69.33)	37 (82.22)	0.39
Positive	23 (30.66)	8 (17.39)	
YAP intensity n (%)			
Citoplasmic YAP			
Negative	30 (40.00)	19 (42.22)	0.97
Low	30 (40.00)	19 (42.22)	
High	15 (20.00)	7 (15.56)	
Nuclear YAP			
Negative	52 (69.33)	37 (82.22)	0.24
Low	8 (10.67)	8 (17.78)	
High	15 (20.00)	0	

chi^2 test. P<0.05.

Table VI

Adjusted odds ratios * for breast cancer and urinary and toenails arsenic concentration.

	OR	95% IC	*P* value
Urinary arsenic metabolites (μg/L)			
As^{III}	0.961	0.805 – 1.145	0.656
As^{V}	1.011	0.881 – 1.161	0.869
MMA^{V}	0.964	0.909 – 1.023	0.234
DMA^{V}	0.994	0.979 – 1.009	0.468
Total As	0.995	0.984 – 1.006	0.432
%iAs	1.011	0.959 – 1.066	0.669
%MMA	0.823	0.706 – 0.958	0.012
%DMA	1.022	0.977 – 1.068	0.333
Arsenic methylation capacity (μg/L):			
Primary methylation	0.637	0.380 – 1.068	0.087
Secondary methylation	1.254	1.008 – 1.559	0.042
Toenails arsenic (μg/g)	0.481	0.232 -1.000	0.050

* Adjusted for age, YAP status, schooling and type of drinking water.