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THE POSSIBILITY OF BENOMYL AND DIAZINON PESTICIDE'S CARCINOGENICITY AND THE POTENTIAL OF HOTAIR AND H19 AS A SERUM BIOMARKER IN BREAST CANCER

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Evidence had grown ever stronger that pesticides can cause epigenetic modifications such as changing the expression of noncoding RNAs which have positive associations with incidence of cancer.

Upregulation of two oncogenic long non coding RNAs, HOX antisense intergenic RNA and H19, enhances breast cancer. This study was conducted to investigate and compare the effect of 7,12-dimethylbenz(a)anthracene (proven to cause breast cancer) with two commonly used pesticides named benomyl and diazinon (suspected of developing breast cancer) on the expression level of HOX antisense intergenic RNA and H19. Mice were intragastrically exposed to 7,12-dimethylbenzathracene, diazinon and benomyl for 60 days. The expression level of H19 and HOX antisense intergenic RNA were measured by Real-Time PCR. The findings revealed that the expression of long non coding RNAs in pesticides and 7,12-dimethylbenzathracene treated mice were significantly higher than untreated control. This study, for the first time, has demonstrated that diazinon and benomyl pesticides could cause upregulation of both oncogenic H19 and HOX antisense intergenic RNA. Since 7,12-dimethylbenz(a)anthracene induced breast tumors, similar results of all three experimental groups could be a testimony to the carcinogenicity of these pesticides and provides support for the importance of these noncoding RNAs as a target for therapeutic intervention in breast cancer.

Keywords: Pesticide, Biomarker, Breast cancer, H19, HOTAIR

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INTRODUCTION

For a woman, breast cancer could be the main cause of cancer-related deaths over the course of her lifetime. Breast cancer impacts 2.1 million women each year and it is estimated that 627,000 women died from breast cancer in 2018 (MEO, 2018).

Although pesticides help human to control the agricultural pests but many generations of them have been found to be harmful for the environment and also for human health. Numerous studies revealed the correlation between pesticide's exposure and hormone-dependent cancer risks such as breast cancer (MNIF *et al*., 2011).

Diazinon is a commonly used organophosphate pesticides (OPPs). Many studies have shown that OPPs induce malignant transformation of breast cells by altering p53 and c-Ha-ras (CALAF *et al*., 2009), accelerates the epithelium of mammary gland in the process of carcinogenesis (CABELLO *et al.*, 2001). However, the aforementioned articles, in particular, did not mention diazinon and carbaryl on their cancer topic. Also a benzimidazole fungicide, benomyl, is a potential endocrine disruptor and is responsible for greater aromatase activity. On the other side, epigenetic modifications such as aromatase activity or cell apoptosis pathways could increase the breast cancer on clinical trial examinations (MITRA and DASH, 2018; PRITCHARD, 2017). So by connecting these two facts together, we expect the benomyl as a risk factor for breast cancer.

Cancer biomarkers could help to detect the cancer early enough which can assist doctors for the treatment in advase.Long non-coding RNAs (long ncRNAs, lncRNA) transcripts longer than 200 nucleotides that are mostly not translated into protein. Recent studies demonstrated that lncRNAs involves in variety of biological processes like epigenetic processes, transcriptional regulation, hormone receptor pathways, RNA precursor and cancer (CALLE *et al*., 2018; RAFIEE *et al*., 2018; YAU *et al*., 2018). The misexpression of lncRNAs has been shown in different kinds of human cancers worldwide. HOTAIR and H19 have oncogenic functions in breast cancer, increasing invasiveness, tumorigenesis and metastasis or downregulate tumor suppressors (RAFIEE *et al*., 2018).

Expression of H19 is higher in Estrogen receptor (ERα) positive cancer cells. H19 induced steady activation of Akt and is a precursor of miR-675 and miR-675 expression. Therefore, enhances breast cancer and cell migration. Upregulation of H19 facilitates S-phase cycle transition G1/S and breast cancer proliferation (COLLETTE *et al*., 2017).

HOTAIR (HOX transcript antisense RNA) is a proto-oncogene and is considered as a marker in breast cancer because it can activates c-Myc, epithelial-to-mesenchymal transition (EMT) and inhibition of mir-17 which led to increase cell invasion and metastasis (PAWŁOWSKA *et al*., 2017).

This study appears to be the first study to compare the effect of 7,12-dimethylbenz(a)anthracene (which induced breast cancer) and two pesticides named benomyl and diazinon (suspected of developing breast cancer) on the expression level of two proto-oncogene H19 and HOTAIR. These lncRNAs could be attractive targets for therapeutic intervention and as a diagnostic serum biomarker in breast cancer.

MATERIAL AND METHODS

Chemicals, mice and study design

A total of 40 female *Mus musculus* (BALB/c) from Production Complex of Pasteur Institute of Iran at Karaj, were classified in to 4 groups (8 mice per group). Pesticides (benomyl and diazinon) were purchased from flower shop (Karaj, Iran). Pesticides and carcinogen were dissolved in corn oil and administered intragastrically by gavage needle for consecutive 60 days. The amounts used are as follows: control group (first group) received 0.9% normal saline, second group received 1 mg/day diazinon (85% wettable powder formulated), third group received 1 mg/day benomyl (85% wettable powder formulated) and forth group received 1 mg/week 7,12-dimethylbenz(a) anthracene (DMBA). All mice had the average weight of 35 gr and 8 weeks old. At weeks 8 post gavage, 32 mice (n = 8 per group) were sacrificed. Blood samples were drawn from their heart and the sera were collected by centrifugation at 5500 r/min for 10 min.

Table 1. A summarized of experimental model

DMBA (carcinogen)	DMBA (carcinogen)	Benomyl (pesticide)	Diazinon (pesticide)	Placebo-controlled group	Experimental series
1 mg/week	1 mg/week	1 mg/day	1 mg/day	0.9% normal saline	Dose (mg)
8 / 8 weeks	8 / 8 weeks	8 / 8 weeks	8 / 8 weeks	8 / 8 weeks	No. of mice / gavage duration
13 weeks after initial administration, mammary tumors detected, then these 8 mice were killed by Chloroform.		32 mice were sacrificed after 8 weeks and their sera were collected			Remarks

Meanwhile, to ensure that the mice will get mammary tumors by DMBA, there were also 8 other mice (in addition to the groups above) that received 1 mg/week DMBA diet for 8 weeks, similar to the mentioned group above. These mice were examined weekly for the appearance of tumors. 13 weeks after initial administration of DMBA, two mice were detected with mammary tumors. These mice then were killed by Chloroform. The experimental model is summarized in Table 1.

RNA extraction

From serum of samples, total RNA was extracted with RNX-PLUS reagent (Cinnagen, Iran) following the manufacturer's instructions. Then 200 µl chloroform was added for removing proteins and cell debris. The upper phase contained RNA was precipitated by adding isopropanol in1:1 ratio and 75% ethanol.

RNA purity was determined with Single beam scanning UV-Vis spectrophotometer M501, measuring A_{260nm}/A_{280nm} ratio (acceptable when the ratio was >1·8).

Reverse transcription

RNA-primer mixture was prepared as follows: 1 µg of RNA, 0.5 µl oligo(dT), 0.5 µl of random hexamer primers and DEPC-treated water up to10µl were incubated at 65°C for 5 minutes and chilled on ice for 2 minutes. Then cDNA synthesis mixture including 2µl of 10X Buffer M-MuLV, 0.5µl M-MuLV Reverse Transcriptase, 0.5µl(20u) RNase inhibitor, 2µl 10mM dNTP Mix and DEPC-treated water up to 10µl were added to the RNA-primer mixture. Then incubated at 25°C for 10 min, 42°C for 60 min and for 5 min at 85°C in a final volume of 20 µl. Reverse transcription was performed in a thermocycler (Exicycler™ 96 Real-Time Quantitative Thermal block, Bioneer).

Quantitative RT-PCR (RT-qPCR)

Real-time PCR amplifications were performed by using a RealQ Plus 2x Master Mix Green kit (Ampliqon). In brief, 2 µl of cDNA, 0.5 µL forward primer, 0.5 µl reverse primer, 12.5 µl RealQ Plus 2x Master Mix and nuclease-free H2O up to 25 µl were mixed. The subsequent three-step PCR program conditions consisted of 1 cycle of 95°C or 15 m, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The following primer sets were used: For HOTAIR: 5´-GGCGGATGCAAGTTAATAAAAC-3´(forward), 5´-TACGCCTGAGTGTTCACGAG-3´ (reverse): product size=92 bp. For H19: 5′-TGTAAACCTCTTTGGCAATGCTGCC-3′ (Forward), 5′-TATTGATGGACCCAGGACCTCTGGT-3′ (Reverse): product size: 111, For (reference gene) hypoxanthine guanine phosphoribosyl transferase (Hprt): 5´-CTCAACTTTAACTGGAAAGAATGT-3´: product size=99 bp.

The relative levels of each lncRNA in mice serum was normalized by Hprt. CT is the threshold cycle to detect fluorescence and ΔCt for each gene is calculated by the formula Δ*Ct* test=*Ct test–Ct* hprt and Δ*Ct* control =*Ct control–Ct* hprt. The relative expression level of lncRNAs to the expression in control, was calculated using $RQ= 2^{-}\Delta\Delta^{CT}$ calculation methodology recommended by Pfaffl. In which ΔΔCT was calculated by the formula $\Delta\Delta C_T= (C_{T\text{lncRNA}}-C_{T\text{Hprt}})_{\text{test}} (C_{T\text{lncRNA}}-C_{T\text{Hprt}})_{\text{control.}}$

Statistical analysis

SPSS software and Graphad Prism (Prism 7.0 Graphpad Software Inc., La Jolla, USA) were applied to test the normal distribution of the results. Parametric unpaired t-test were used for multiple comparisons. Data are presented as mean ± SEM. *P*-values <0.05 were considered statistically significant.

RESULTS

Real-time PCR analysis to compare the expression level of H19 and HOTAIR between test and control groups

32 mice that were classified into 4 groups, including control and 3 other groups gavaged with benomyl, diazinon and DMBA. These mice were sacrificed after 8 weeks. Quantitative real-time PCR was used in order to analyze the expression level of H19 and HOTAIR in serum. H19

and HOTAIR expression level ($2^{-\Delta\Delta CT}$) in all treatment groups was significantly higher (*P < 0.05) compared with the control group during all two months of experiment.

Mean expression level of H19 in diazinon, benomyl and DMBA treated groups on the 60th day was 3.03 (SEM ±0.5), 8.7 (SEM ±0.9) and ≥ 11.3 (SEM ±1.3) respectively. The mean expression of control group was 1.1 (Fig. 1).

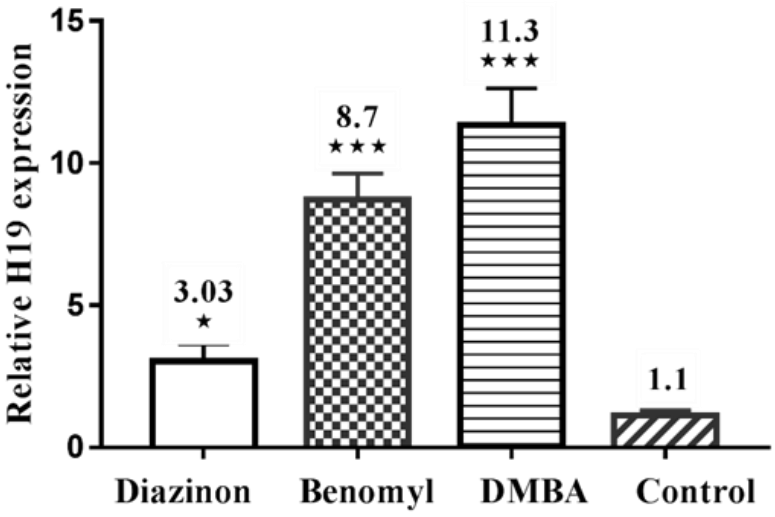

Fig. 1. Effects of benomyl, diazinon and DMBA on the expression of H19-lncRNA in *Mus musculus* serum by RT-qPCR assay. Y-axis represents the mean of $RQ= 2^{-\Delta\Delta CT}$. Bars represent the standard error of the mean. Each value on the column represent mean expression level of H19-lncRNA (n=8), *P < 0.05 compared with control group (unpaired t test). The expression profile of H19 showed that the mean expression of H19 was significantly more in treated groups compared with control group (*P<0.05) on the day 60th.

The results also showed that H19 level was significantly overexpressed to ≥ 2.7-fold, ≥ 7.8-fold and ≥ 10.2-fold in diazinon, benomyl and DMBA treated groups respectively after 8 weeks, compared with the control group.

Mean expression level (*P < 0.05) of HOTAIR in diazinon, benomyl, DMBA and control groups was also determined by 2.6 (SEM ±0.4), 6.8 (SEM ±0.1), 9.2 (SEM ±0.1) and 1.1 (SEM ±0.2) after 8 weeks (Fig. 2). Upregulation of HOTAIR was ≥ 1.9-fold, ≥ 4.9-fold and ≥ 6.6-fold respectively in diazinon, benomyl and DMBA treated groups compared with the control group.

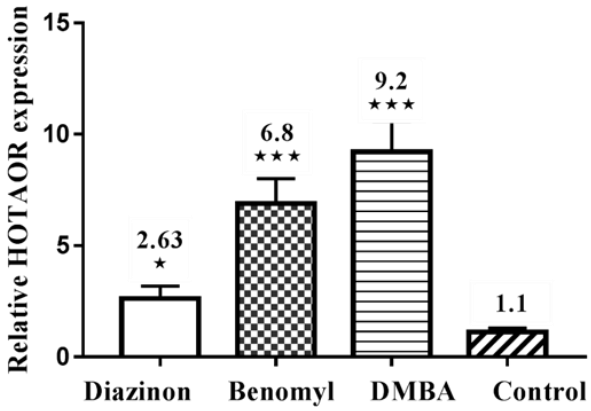

Fig. 2. Effects of benomyl, diazinon and DMBA on the expression of HOTAIR- by RT-qPCR assay. The expression profile of HOTAIR in treated and control groups on the day 60th. Y-axis represents the mean of $RQ = 2^{-\Delta\Delta CT}$. Bars represent the standard error of the mean. Each value on the column represent relative expression of HOTAIR -lncRNA (mean± standard deviation; n=8). *P < 0.05 compared with control group (unpaired t test). The results showed that the mean expression of HOTAIR was significantly higher in benomyl, diazinon and DMBA groups compared with control on the day 60th.

DISCUSSION

Breast cancer is the most serious invasive malignancy besides skin cancer and allocate 15% of all cancer deaths among women and there have been many studies in genetics and molecular biology leading to ameliorate its diagnosis and therapy (MEO, 2018).

Several pesticides can increase estrogen production or have endocrine-disrupting properties which can interfere with the synthesis and metabolism of estrogen and influence on breast cancer risk (NIEHOFF *et al*., 2016). For example, malathion, benomyl and diazinon probably increase the risk of breast cancer by acting like endocrine disruptors (FENGA, 2016; YAU *et al*., 2018).

Biomarkers such as non-coding RNAs are beginning to play an important role as a prognostic indicator across all human cancers. lncRNAs can be important regulators in basal-like breast cancer (BLBC). The study examined BLBC-specific lncRNA, BLAT1, expression in a twenty breast cancer cell lines showed that breast cells have higher expression of BLAT1 in general (HAN *et al*., 2018).

The overexpression of H19 was able to upregulate the expression level of active RAS-MAPK signaling pathway in colorectal cancer (CRC) cells, promoting cell migration and invasion (YANG *et al*., 2018). H19 is also believed to involve in transcriptional and posttranscriptional regulation, tumor suppression and oncogenesis specially in breast cancer (BERTEAUX *et al*., 2008). H19 is overexpressed in 70% of human breast cancer. H19 overexpression increases cells invasion, cell cycle progression, cell invasion, angiogenesis and induce overexpression of Hepatocyte growth factor (HGF) growth factor. Also, H19 is the

precursor of miR-675-5p which targets Cbl-b and c-Cbl mRNA in breast cancer. Downregulation of Cbl-b and c-Cbl ubiquitin-protein ligase expression induces activation of Akt and Erk cell signalling that promotes survival, growth and migration potential (VENNIN *et al*., 2013; VENNIN *et al*., 2015).

Another lncRNA named HOTAIR interacts with matrix metalloproteinases (MMPs). MMPs are capable of degrading extracellular matrix proteins which can help cancer progression, migration, angiogenesis and invasion (PAWŁOWSKA *et al*., 2017). PRC2 (polycomb repressive complex 2) has histone methyltransferase activity and is required for epigenetic silencing of chromatin. HOTAIR lncRNA can target PRC2, alter methylation and gene expression patterns, and increased breast cancer metastasis (TSAI *et al*., 2011).

The structural modelisation of the 200 nt of the 3″ region of HOTAIR showed that it interacts directly to proteins through its 3″ end and inhibits tumor suppressor ones like P53, PRC2 EGFR and STAT55. Therefore, HOTAIR can induce breast cancer development (LAMSISI *et al* ., 2018).

HOTAIR expression analysis in breast invasive carcinoma tissues derived from TCGA (The Cancer Genome Atlas) showed that the HOTAIR is overexpressed in breast tumors (n= 1,066) compared to normal tissues (n= 133) (Avazpour et al., 2017).

In the present study, for the first time, we assessed the effect of DMBA (which induce breast cancer) and two pesticides named benomyl and diazinon (suspected of developing breast cancer) on the expression level of two proto-oncogene H19 and HOTAIR.

Pesticides and carcinogen were administered intragastrically for 60 days. At weeks 8 post gavage, mice were sacrificed. In order to investigate whether pesticides (diazinon, carbaryl) and DMBA can induce lncRNAs dysregulation, we analyzed H19 and HOTAIR expression level in the sera of BALB/c mice by real-time PCR method.

Meanwhile, to ensure that the mice will get mammary tumors by DMBA carcinogen, 8 other mice (in addition to the groups above) that received DMBA for 8 weeks were not sacrificed. These mice were examined weekly for the appearance of tumors. 13 weeks after initial administration of DMBA, mammary tumors were detected.

Real-time PCR results showed that diazinon, benomyl and DMBA treated groups had higher expression of H19 than control group which was $\geq$ 2.7-fold, $\geq$ 7.9-fold and $\geq$ 10.2-fold respectively). Upregulation of HOTAIR for diazinon, benomyl and DMBA after 60 days was by $\geq$ 2.3-fold, $\geq$ 6.1-fold and $\geq$ 8.3-fold respectively compared with the control group.

Since DMBA induced breast tumors after 13 weeks post gavage, similar higher expression results of oncogenic H19 and HOTAIR in benomyl, diazinon and DMBA treated groups could be a testimony to the potential of these pesticide's carcinogenicity. Also, it provides support for the importance of these lncRNAs as a biomarker in breast cancer.

The findings revealed that the expression level of HOTAIR and H19 lncRNAs in pesticide groups were consistent with DMBA group, so that all treated mice had significantly higher expression compared with the untreated control during two months.

The present study, for the first time, has demonstrated that diazinon and benomyl pesticides could cause upregulation of both H19 and HOX antisense intergenic RNA. These findings highlight the possibility of benomyl and diazinon pesticide's carcinogenicity and the

potential of HOTAIR and H19 as a serum biomarker in breast cancer. Therefore, these lncRNAs could be attractive as targets for therapeutic intervention in cancer.

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MOGUĆNOST KARCINOGENOSTI PESTICIDA BENOMILA I DIAZINONA I POTENCIJAL HOTAIRA I H19 KAO BIOMARKER SERUMA KOD RAKA DOJKE

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Izvod

Sve je više dokaza da pesticidi mogu da izazovu epigenetske modifikacije kao što je promena ekspresije nekodirajućih RNK koje imaju pozitivnu povezanost sa učestalošću raka. Ova studija je sprovedena kako bi se istražilo i uporedilo dejstvo 7,12-dimetilbenz (a) antracena (dokazano izaziva rak dojke) sa dva često korišćena pesticida pod nazivom benomil i diazinon (za koje se sumnja da razvijaju rak dojke) na nivo ekspresije HOKS antisense intergene RNK i H19. Miševi su bili intragastrično izloženi 7,12-dimetilbenzatracenu, diazinonu i benomilu tokom 60 dana. Nivo ekspresije H19 i HOKS antisense intergene RNK meren je RT-PCR-om. Nalazi su otkrili da je ekspresija dugih nekodirajućih RNK u miševima tretiranim pesticidima i 7,12-dimetilbenzatracenom značajno veća od netretirane kontrole. Ova studija je po prvi put pokazala da diazinon i benomil pesticidi mogu prouzrokovati regulaciju kako onkogenog H19, tako i HOKS antisense intergene RNK. Budući da su tumori dojke izazvani 7,12-dimetilbenz (a) antracenom, slični rezultati sve tri eksperimentalne grupe mogli bi biti svedočanstvo o kancerogenosti ovih pesticida i pružaju podršku značaju ovih nekodirajućih RNK za terapijsku intervenciju u raku dojke .

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