ALTERATIONS OF EXPRESSION OF RNA MODIFICATION REGULATORS BY CARCINOGENS IN THE ALTERNATIVE CHICKEN EGG MODEL

Kobets T[1], Duan JD[1], Brunnemann KD[1], Iacobas DA[2], Iacobas S[1], Vock E[3], Deschl U[3], Williams GM[1]

[1] New York Medical College, Valhalla, NY, USA [2] Prairie View A&M University, Prairie View, TX, USA [3] Boehringer Ingelheim Pharma GmbH&Co. KG, Biberach an der Riss, Germany

ABSTRACT

The abundance of cellular RNA structural modifications is well documented. Such epitranscriptome alterations ultimately regulate the expression of genes that control many biological processes. However, their role in the development of many pathologic conditions, including chemical carcinogenesis in the developing organism, remains unclear. Similar to epigenetic alterations, the dynamic nature of epitranscriptome modifications is controlled by various enzymes, including methyltransferases, pseudoU synthetases and demethylases. The current study utilized microarray platform to investigate presence and expression of genes which encode for the aforementioned enzymes in the Chicken Egg Model (CEM) after 3 daily injections of a wide array of established genotoxic and epigenetic carcinogens and their comparators. The set of tested chemicals included dialkylnitrosamines, aromatic amines, polycyclic aromatic hydrocarbons, aflatoxins, clofibric acid and phenobarbital. The CEM is an alternative to animal models tool that uses fetal chicken livers collected 3 hours after the last dosing on the incubation day 11, to evaluate various effects of chemicals, including their potential to produce DNA damage, alterations in gene expression and histologic changes. Chicken embryo-fetus is an intact, metabolically active organism, which by definition is not yet subjected to regulations as an animal. Chemical-specific deregulation of 15 genes which encode for several RNA modification enzymes in other species including humans, was observed in CEM. These include *METTL14, ALKBH5, FTO, PUS, TRMT, ALKBH3* and *TET.* Most significant changes in the gene expression pattern were produced by genotoxic hepatocarcinogens dialkylnitrosamines and benzo[a]pyrene. Moreover, the majority of genotoxic carcinogens also altered the expression of several small nucleolar RNAs, including SNORD17, SNORA62 and SNORA81, which guide methylation and pseudo-uridylation of rRNA and tRNA. These findings lead to the hypothesis that chemicals are capable of producing epitranscriptome modifications, which in combination with established genotoxic and/or epigenetic DNA damaging effects contribute to carcinogenesis. Based on our findings, CEM is an appropriate model to investigate this hypothesis.

INTRODUCTION

Methylation of RNA bases, was first observed in eukaryotic cells, including rat and human cancer cells. A plethora of post-transcriptional chemical modifications of various RNA species has been established since. Epitranscriptome modifications play an important role in stability, processing, export and translation of mRNA and maintenance of RNA structure. These changes contribute to regulation of gene expression and control many biologic and developmental processes, including cell differentiation. RNA modifications are dynamic, and as such, are controlled by various enzymes (Fig. 2).

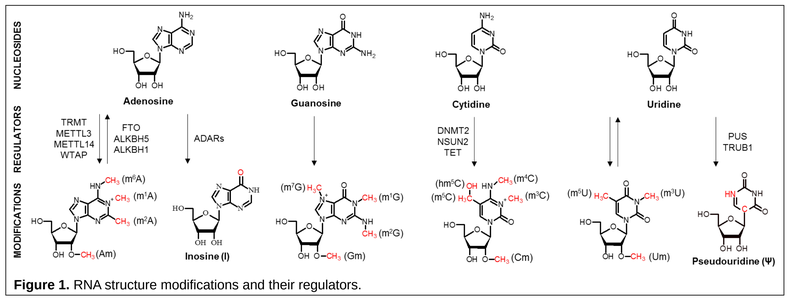

Figure 1. RNA structure modifications and their regulators.

Chicken Egg Model (CEM) is an alternative to animal model that allows for extensive evaluation of multiple effects produced by xenobiotics, including genotoxicity, teratogenicity, histopathologic changes and genomic profiling. Thus, this novel model can be utilized to assess chemical-induced epitranscriptome modifications in the liver tissue of intact, metabolically competent chicken embryo-fetuses.

MATERIALS AND METHODS

Chemicals (Fig. 2) and Solutol HS15 were obtained from Sigma-Aldrich (St Louis, MO). Deionized water was prepared with a Picopure System (Hydro Services and Supplies, Garfield, NJ).

Dialkylnitrosamines	Aromatic Amines	Aromatic Hydrocarbons	Aflatoxins	Epigenetic Carcinogens
Nitrosodiethylamine (2 mg/egg)	2-Acetylaminofluorene (0.6 mg/egg)	Benzo[a]pyrene (0.5 mg/egg)	Afatoxin B1 (1.6 µg/egg)	Phenobarbital (20 mg/egg)
N-nitrosodiethanolamine (4 mg/egg)	Fluorene (1.4 mg/egg)	Benzo[e]pyrene (0.5 mg/egg)	Afatoxin B2 (6.4 µg/egg)	Clofibric acid (1.5 mg/egg)

Figure 2. Set of chemicals tested in the study. Dose levels in parenthesis represent total dosages administered in 3 daily injections.

Experimental design is described in detail in Williams *et al.* (2014).

RNA extraction and gene expression analysis is described in detail in Iacobas *et al.* (2013) and Kobets *et al.* (2018). GOEAST software was used for the Gene Ontology analysis.

^{32}P-postlabeling and thin layer chromatography (TLC) was conducted according to the protocol (Bodi and Fray, 2017).

Statistical analysis was conducted using paired t-tests with Bonferroni correction for multiple testing. *p*-values < 0.05 were considered significant.

RESULTS

Table 1. Expression of established regulators of RNA modifications in the livers of 11-day chicken fetuses exposed to xenobiotics.

Modification	Role	Gene ID	DEN[a]	NDELA[a]	AAF[b]	FLU[b]	BaP[b]	BeP[b]	AFB$_1$[b]	AFB$_2$[b]	PB[a]	CFA[b]
			CHEMICALS									
m^{6}A	W	METTL14										
	E	ALKBH5										
		FTO										
m^{1}A	W	TRMT10A										
		TRMT10C										
		TRMT12										
		TRDMT1										
	E	ALKBH3										
hm^{5}C	W	TET3										
Ψ	W	PUS1										
		PUS10										
		PUS7										
		PUS7L										
		PUSL1										
		TRMU										

Tab. 1. Dark red and dark green colors indicate significantly ($p \leq 0.05$) up or down-regulated (FC > 1.5 or < -1.5) genes, respectively. Pink and light green colors indicate not significantly deregulated genes. Yellow color indicates expression not quantified in all replicas. W, writer, E, eraser. a, vehicle deionized water; b, vehicle 20% aqueous solution of HS15

Table 2. Expression of snoRNAs in the livers of 11-day chicken fetuses exposed to xenobiotics.

Rfam prediction	Host gene	DEN[a]	NDELA[a]	AAF[b]	FLU[b]	BaP[b]	BeP[b]	AFB$_1$[b]	AFB$_2$[b]	PB[a]	CFA[b]
		CHEMICALS									
SCARNA6	*APG16-like*										
SNORD17	*DKC1*										
Unclassified (Ggn68)											
SNORA62	*RPSAP58*										
SNORA81	*EIF4A2*										

Tab. 2. Dark red and dark green colors indicate significantly ($p \leq 0.05$) up or down-regulated (FC > 1.5 or < -1.5) genes, respectively. Pink and light green colors indicate not significantly deregulated genes. a, vehicle deionized water; b, vehicle 20% aqueous solution of HS15.

Table 3. Expression of genes potentially involved in the epitranscriptome regulators in the livers of 11-day chicken fetuses exposed to xenobiotics.

Gene ID	DEN[a]	NDELA[a]	AAF[b]	FLU[b]	BaP[b]	BeP[b]	AFB$_1$[b]	AFB$_2$[b]	PB[a]	CFA[b]
	CHEMICALS									
Methyltransferases										
METTL10										
METTL11A										
METTL13										
METTL15										
METTL16										
METTL18										
METTL21A										
METTL21D										
METTL22										
METTL2A										
METTL5										
METTL6										
METTL8										
METTL9										
tRNA methyltransferases										
TRMT11										
TRMT13										
TRMT1L										
TRMT2A										
TRMT6										
TRNT1										
Demethylases										
ALKBH2										
ALKBH8										

Tab. 2. Dark red and dark green colors indicate significantly ($p \leq 0.05$) up or down-regulated (FC > 1.5 or < -1.5) genes, respectively. Pink and light green colors indicate not significantly deregulated genes. Yellow color indicates expression not quantified in all replicas. a, vehicle deionized water; b, vehicle 20% aqueous solution of HS15.

RESULTS

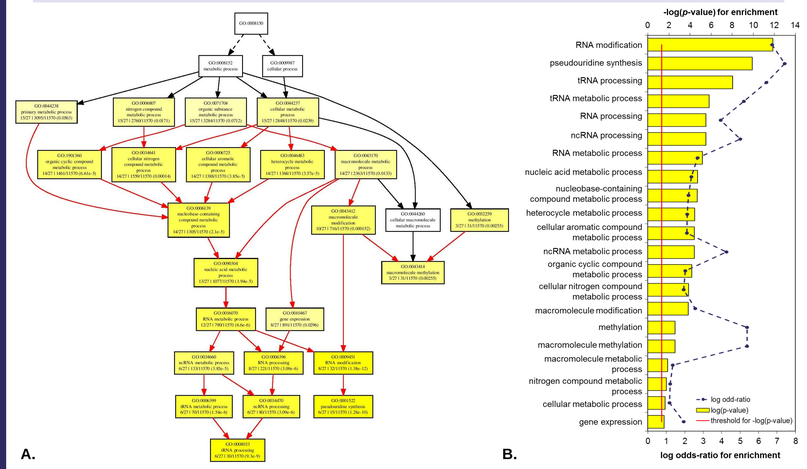

Figure 3. Functional analysis of selected genes according to the Biological Process category of Gene Ontology. Enriched GO terms in the hierarchical tree (**A**) are shown in yellow boxes, non-enriched in white. The *p*-values and ratio for pathway enrichment are shown in the chart (**B**).

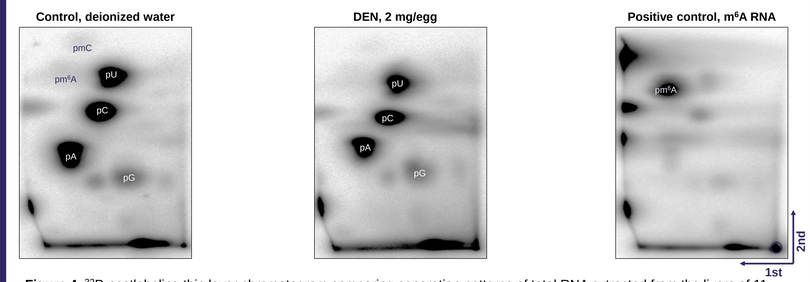

Figure 4. ^{32}P-postlabeling thin layer chromatogram comparing separation patterns of total RNA extracted from the livers of 11-day chicken fetuses exposed to nitrosodiethylamine (DEN) and vehicle control after T1 and T4 digestion. m^{6}A RNA purchased from Epigentek Group Inc. (Farmingdale, NY) was used as a positive control. pA, adenosine 5′ monophosphate; pC, cytosine 5′ monophosphate; pU, uridine 5′ monophosphate; pG, guanosine 5′ monophosphate. pGs are underrepresented in all samples as T1 cuts after every G. The purple circle indicates the site of sample loading. The purple letters are modified nucleotides: pm^{6}A is N^6-methyladenosine 5′ monophosphate, pmC, 2′-O-methyl-cytosine 5′ monophosphate.

CONCLUSIONS

- **In fetal chicken livers exposure to xenobiotics (mainly established genotoxic hepatocarcinogens dialkylnitrosamines) altered expression of genes known to regulate RNA modifications**
- **Nitrosamines, as well as genotoxic hepatocarcinogens 2-acetylamino-fluorene and benzo[a]pyrene also affected expression of several small non-coding RNAs that guide chemical RNA modifications.**
- **Additional genes that can be potentially involved in the epitranscriptome regulation were detected.**
- **Low levels of modified RNA nucleotides (m^{6}A) in the total RNA from fetal chicken livers were captured in the preliminary study using TLC method.**
- **These findings support a hypothesis that epitranscriptome alterataions could play an important role in the process of chemical carcinogenesis.**
- **Overall, Chicken Egg model can be used to investigate this hypothesis. The detection of modified nucleotides can be significantly improved using high throughput sequencing methods.**

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The authors have no conflict of interest to disclose