Role of Oxidative Stress in Prognosis of Ovarian Cancer

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Ovarian cancer is one of the most lethal gynecological malignancies. Mitochondria are the predominant source of reactive oxygen species (ROS) in the cell. Besides mitochondria nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) enzymes generate a significant amount of ROS in the cell. The present work establishes an interesting link between NOX4 enzyme (which is an important source of reactive oxygen species "ROS") and PHB1 (as a holdase type chaperone in mitochondrial stress). The current study was conducted on 60 patients with ovarian tumours (benign, borderline and malignant) and 20 healthy volunteers (as a control group). *NOX*4 expression was assessed by TaqMan® real time gene expression assay, while cellular expression of prohibitin was evaluated by immunohistochemistry. There was a significant increase in prohibitin expression from benign cystadenoma to malignant tumors. In addition, there was an increase in *NOX*4 expression. In conclusion, over-expression of PHB1 and *NOX4* in malignant ovarian tissues suggest that PHB1 is associated with tumorigenesis via activation of NOX4 enzyme with subsequent release of ROS in the cells.

Ovarian cancer is one of the most lethal gynaecological malignancies. Usually more than two thirds of the patients present at advanced stage and have an overall 5-years survival of 30%. Hence, the poor prognosis could be improved by a better understanding of its molecular pathogenesis, which may ultimately contribute to the identification of new biomarkers useful for its early detection, and development of therapies [1].

King (2013) [2] has reported that "clinical and epidemiological investigations have provided evidence supporting the role of reactive oxygen species (ROS) in the prognosis and metastasis of cancer due to exogenous factors that lead to chronic inflammation. Cancer cells are frequently under persistent oxidative stress which participates in cancer progression as well as the selection of resistant cells that cannot be eliminated by apoptosis"[2].

Oxidative stress is defined as an imbalance between excessive ROS "oxidants" and the anti-oxidant defense system. This imbalance influences the redox state of the cells [3]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and mitochondria are important sources of ROS in the cell. Nicotinamide adenine dinucleotide (NAD+) is an essential electron transporter in mitochondrial respiration and oxidative phosphorylation. NOX oxidase enzymes catalyze the NADH dependant reduction of molecular oxygen to superoxide and other ROS. Mutations of specific regions of NAD(P)H oxidase cause enhancement of the enzyme activity that lead to an increase in superoxide $O2^{\bullet}$ production (oxidative stress) [4, 5]. Thus,

mitochondria are extremely susceptible to endogenous ROS. In addition, mutation in mitochondrial DNA has tumour promoting effects due to the increased levels of mitochondrial ROS resulting from the escape from the respiratory chain when these genes' products have increased functions [4].

Prohibitins (PHB), a highly conserved group of proteins, are expressed in many cell types and are mainly located in the mitochondria, nucleus and plasma membrane [6]. PHB binds directly to the newly synthetized mitochondrial translation products to protect them from degradation. The best described function being a chaperon protein involved in the stabilization of mitochondrial respiratory enzymes [7, 8]. Membrane PHB regulates the cellular signaling of membrane transport. Nuclear PHB controls transcription and cell cycle. Mitochondrial PHB complex stabilizes the mitochondrial genes and modulates mitochondrial morphology and dynamics as well as the intrinsic apoptotic pathway [9-11].

Sripathi, *et al.*, (2011) [12] have postulated that PHB is a transport protein that shuttles between the subcellular compartments. Peng, *et al.*, (2015) [6] have suggested that subcellular localization of PHB may determine its functions. They reported that "nuclear shuttling movement of PHB correlates with signal transduction, cellular proliferation, apoptosis and differentiation".

Because mitochondrial DNA encodes for essential subunits of oxidative phosphorylation (OXPHOS) system, regardless whether PHB affects mitochondrial DNA directly or indirectly, the net result will likely be defects in OXPHOS and this will link NOX4 to PHB1 in the present work.

Participants and Methods

Study design

This case control study was conducted on 80 Participants; 60 patients with ovarian tumours from Obstetrics and Gynaecology department, Faculty of Medicine, Alexandria University and 20 healthy females during the period between 2017-2018. Participants were divided into 4 groups; group 1 included 20 patients with benign lesions; 7 females were at menopause while 13 were in pre-menopause state, group 2 included 20 patients with borderline ovarian cancer; 12 females were at menopause while 8 were in pre-menopause state, group 3 included 20 patients with malignant ovarian cancer; 10 females were at menopause and 10 were in pre-menopause state, and group 4 (control group) included age matched healthy females; 10 were at menopause and 10 were in pre-menopause state. Exclusion criteria include secondary ovarian tumors, hepatic or renal decompensation and presence of any autoimmune disease.

Ethics approval and consent to participate

This study was accepted by and following guidelines of the ethical committee of Faculty of Medicine, Alexandria University; according to declaration of Heliniski. All participants were asked to freely volunteer to the study and informed written consents, for patient participation in a clinical research, were gathered from all participants before their inclusion in the study.

Samples collection

Six ml venous blood were obtained from each subject under the study; 3 ml were collected in plain tubes (for serum separation to be used for serological investigations), and 3 ml was put in tubes with EDTA salt (for RNA extraction and molecular assays). Tissue samples from ovarian cancer patients were taken during surgery.

Histopathological examination

Hematoxylin and Eosin (H&E)-stained slides from a total of 60 cases were reviewed, characterized and studied. All malignant cases were diagnosed and graded using the criteria of the International Federation of Obstetrics and Gynecology (FIGO) [13].

Evaluation of NOX4 mRNA expression

NOX4 expression was determined using TaqMan® Gene Expression Assays protocol provided from Applied Biosystems. Total RNA extraction from ovarian tissue samples was done using the PureLink® RNA Mini Kit (cat.≠12183018A, Ambion, Life Technologies). RNA concentration and purity was assessed by a Nanodrop spectrophotometer. The optical density OD260:OD280 ratio of all RNA samples was ranging between 1.8 and 2.0. Reverse transcription of RNA to complementary DNA (cDNA) was carried out using high capacity cDNA reverse transcription kit (Applied Biosystems, Life Technology Company) according to the manufacturer's protocol. Quantitative RT-PCR was done using StepOne™ Real-Time PCR System (Applied Biosystems, Life Technologies) with TaqMan® Gene Expression *NOX4* Assays (TaqMan®, FAM™ dye-labeled) following the manufacturer's instructions. Results were calculated according to the manufacturer's instructions as follow: ΔCT=CT of *NOX4* - CT of house keeping gene (GAPH), ΔΔCT=ΔCT of patient - mean of ΔCT of *NOX4*, relative quantity (RQ)=$2^{-\Delta\Delta CT}$, RQ < 1 (i.e. control) means down-regulation, RQ>1 means up-regulation.

Assessment of prohibitin expression

Tissue sections of ovarian tissues (5 µm) were immunohistochemically (IHC) stained using the purified mouse anti-prohibitin antibody (E-5, sc-377037, Santa Cruz Biotechnology) and the standard avidin-biotin-complex technique was used as described previously [14, 15].

Evaluation of results as positive or negative was done as described by El-Etreby *et al.,* (2017) [16] who stated that "percentage of neoplastic and non-neoplastic cells that showed dark brown cytoplasmic staining was recorded. The staining intensity was graded as: 0=no staining; 1=weak staining; 2=moderate staining; and 3=intense staining. Prohibitin IHC index was generated by multiplying the intensity by the percentage of positive cells in a defined specimen. Prohibitin IHC index yields scores ranging from 0 to 300. Stained cells exceeding 10% were considered positive and staining was performed in duplicates".

Statistical Analysis

Data analysis was done by IBM SPSS software package version 20.0 (Chicago, USA). Qualitative data were described by numbers and percentage. Comparison between the different groups regarding categorical variables was done by Chi-square test. When more than 20% of the cells have expected count below 5, correction for chi-square was done by Monte Carlo correction. Significance was determined at the 5% level [17].

Results

Participant 's demographic data

Age distribution among all the studied groups ranged between 40 to 60 years old. There were not any statistically significant differences between the studied groups regarding age or menstrual state.

Pathologic features of studied cases

H&E staining of the slides from a total of 60 cases have clarified that among the benign ovarian lesions 12 cases were serous cystadenomas and 8 were mucinous cystadenomas as shown in Figure 1a and 1b. Regarding borderline lesions, 15 cases were serous borderline tumours while 5 were mucinous borderline tumours as illustrated in Figure 1c, 1d. The cases of ovarian carcinomas were serous (n=9), mucinous (n=4), endometrioid (n=5), and poorly or undifferentiated (n=2) (Figure 1e-1h). The mucinous carcinomas were all ovarian primary by clinicopathologic studies. Serous lesions were significantly more common than mucinous lesion in all the studied cases of ovarian tumour (*P*=0.009*).

Expression of NOX4mRNA among studied groups

The relative quantitation of NOX4mRNA expression showed a statistically significant over-expression among patients with borderline and malignant ovarian tumours (*P*= 0.001*) (Table 1).

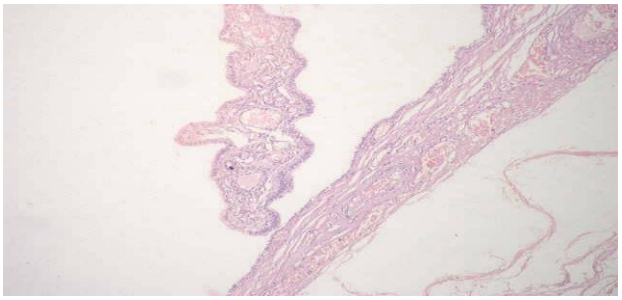

Figure 1a. Papillary serous cystadenoma showing a cyst wall lined by single layer of cuboidal epithelial cells forming simple papillary processes. No stromal invasion (H&E; x10)

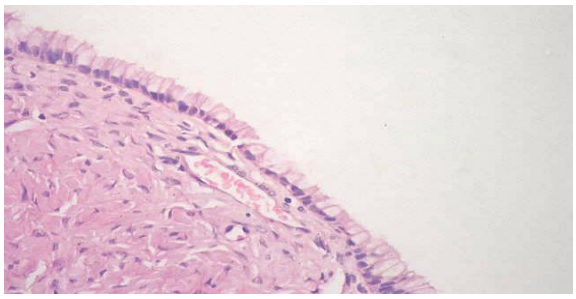

Figure 1b. Benign mucinous cystadenoma. The cyst wall is lined by "picket-fence" columnar epithelium. The cytoplasm is filled with mucin while the nuclei are small & basal. (H&E; x40)

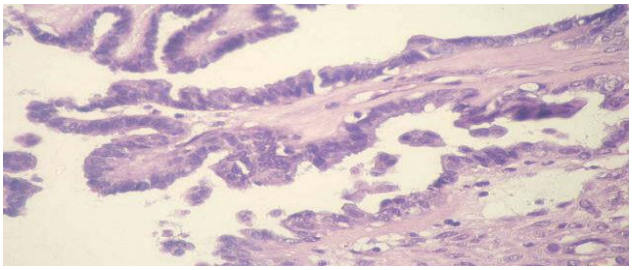

Figure 1c. Borderline serous tumour with papillary projections of epithelium extending into the tumour lumen, without invasion of the stroma. (H&E; x40)

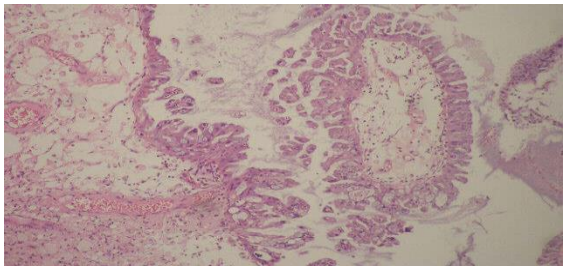

Figure 1d. Borderline mucinous tumour showing papillary structures lined by stratified epithelial cells with marked nuclear atypia. No stromal invasion & background shows mucoid secretion (H&E; x10)

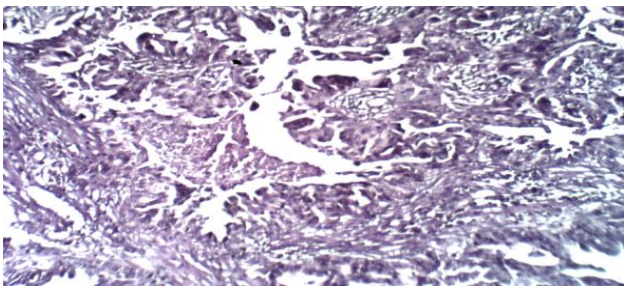

Figure 1e. Papillary serous cystadenocarcinoma showing pronounced papillary growth with stromal invasion by malignant epithelial cells. (H&E; x10)

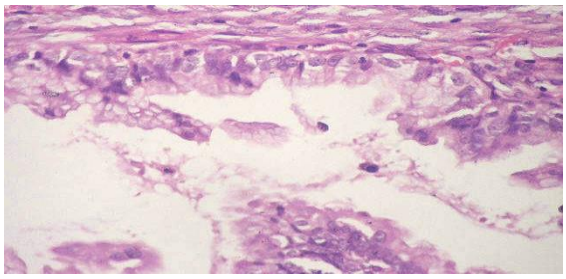

Figure 1f. Mucinous cystadenocarcinoma (cystic and papillary structures lined by stratified malignant epithelial cells with abundant mucin) (H&E; x40)

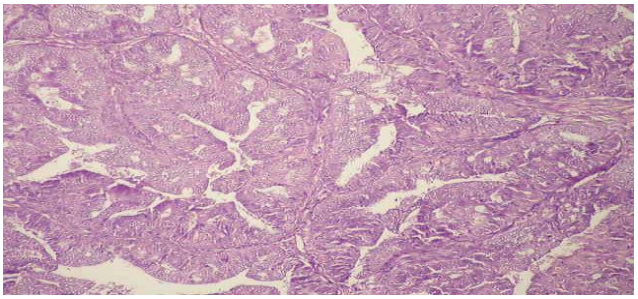

Figure 1g. Endometrioid adenocarcinoma (compact glandular structures lined by stratified malignant epithelial cells) (H&E x 10)

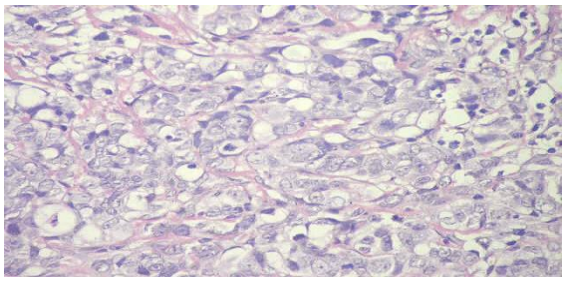

Figure 1h. Poorly differentiated carcinoma shows nesting of high grade malignant epithelial cells. (H&E; x40)

Table 1. *NOX4* expression among the studied groups.

NOX4mRNA expression	Group				^{H}P value
	Control	Benign	Borderline	Malignant	
Minimum	0.007	0.002	0.320	0.020	0.001*
Maximum	0.620	1.102	16.450	29.240	
Mean±SD	0.214±0.256	0.510±0.355	6.166±4.301	5.314±5.583	
Median	0.115	0.364	0.630	0.170	

H: Kruskal-Wallis test, * P<0.05 is significant.

Prohibitin expression among studied groups

Prohibitin expression in the studied ovarian tissues was assessed by IHC. The means of immunostaining scores, summarized in Table 2, showed statistically significant increase in prohibitin expression from benign cystadenoma to malignant tumours (P=0.001*). Figure 2a showed PHB expression in normal ovarian follicle. PHB expression in benign papillary serous and mucinous ovarian tumors is represented in Figures 2b and 2c, respectively. Figures 2d & 2e showed PHB expression in borderline papillary serous and mucinous ovarian tumors, respectively. Figures 2f - 2i show PHB expression in malignant papillary serous, mucinous, endometrioid and undifferentiated ovarian tumors, respectively.

Table 2. Prohibitin expression by IHC among the studied groups.

Prohibitin expression by IHC		Group				^{F}P value
		Control	Benign	Borderline	Malignant	
Intensity	(Mean ± SD)	2.2 ± 0.6	2.0± 0.0	2.6 ^{d}± 0.5	3.0 ^{d}± 0.0	0.001*
%	(Mean± SD)	69.3±10.2	66.3±16	85.6 ^{d}±9.0	96.3 ^{d}±3.9	0.001*
Score	(Mean ± SD)	147.8±43.5	132.5±31.9	226.7 ^{d}±64.4	288.8 ^{d}±11.8	0.001*

F:ANOVA test, * P<0.05 is significant.

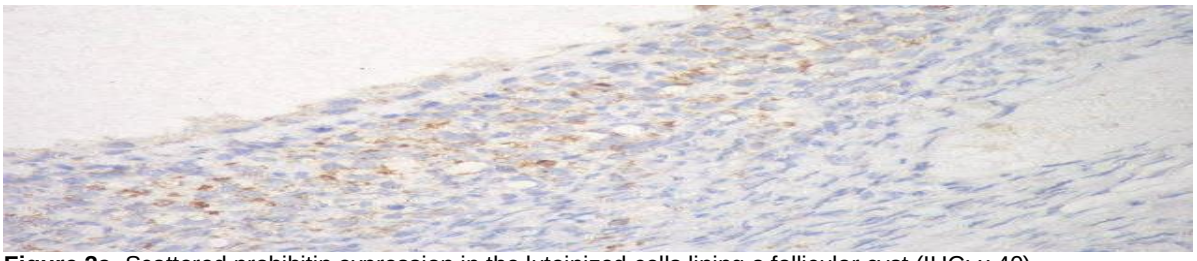

Figure 2a. Scattered prohibitin expression in the luteinized cells lining a follicular cyst (IHC; x 40)

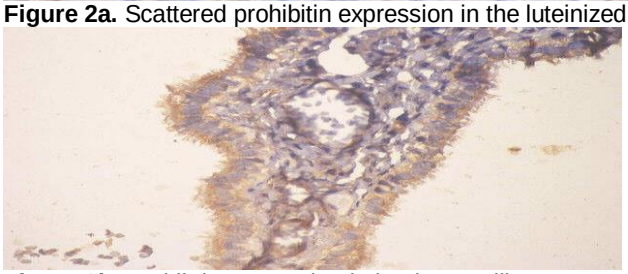

Figure 2b. Prohibtin expression in benign papillary serous cystadenoma. (IHC; x40)

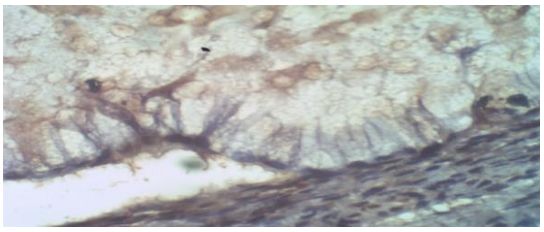

Figure 2c. Weak prohibitin expression in benign mucinous cystadenoma.(IHC; x40)

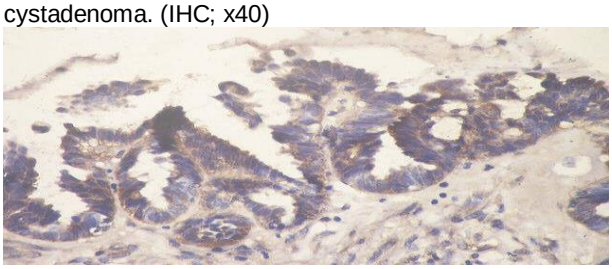

Figure 2d. Moderate prohibtin expression in border-line serous tumour. (IHC; x40)

Figure 2e. Moderate expression of prohibitin in borderline mucinous tumour. (IHC x40)

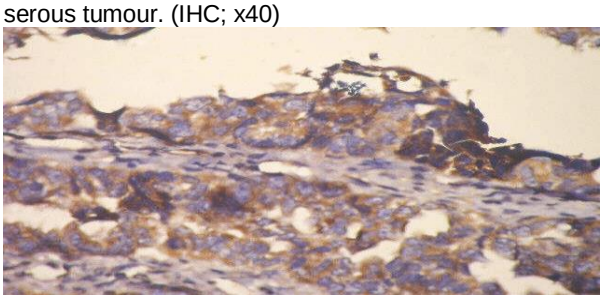

Figure 2f. Strong prohibitin expression in malignant papillary serous cystadenocarcinoma. (IHC; x40)

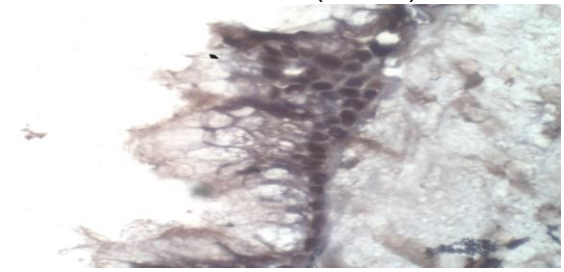

Figure 2g. Prohibitin expression in a case of mucinous cystadenocacinoma. (IHC; x40)

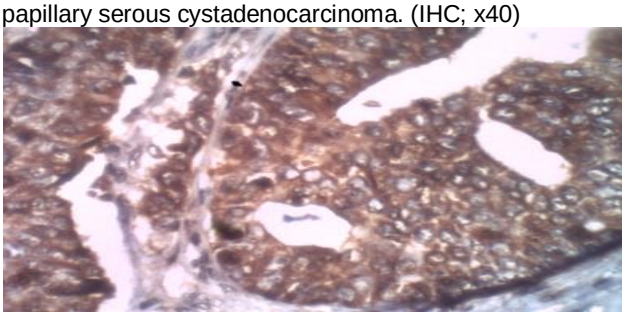

Figure 2h. Strong prohibitin expression malignant endometrioid adenocarcinoma (IHC; X 40)

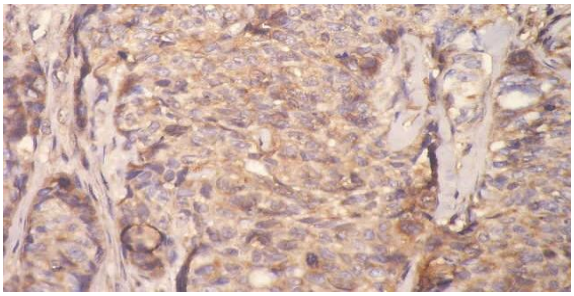

Figure 2i. Strong prohibitin expression in malignant poorly differentiated carcinoma. (IHC; x40)

Discussion

Ovarian cancer is one of the leading causes of cancer-related deaths among all gynecological malignancies. However, the precise biologic mechanisms of ovarian epithelial carcinogenesis are poorly understood [18]. This study highlights the critical roles of *NOX4* and PHB1 in the prognosis of ovarian cancer.

Expression of *NOX4* enzymes in our results suggests an important role of *NOX4* family in increasing tumourigenic potential. The current data revealed that over 70% of ovarian tumours express *NOX4*. Many studies have supported our results regarding *NOX4* regulation of tumourigenesis [19, 20]. *NOX4* over-expression was also correlated with P38MAPK pathway. *NOX4* might be an active enzyme sensor relaying on P22PHOS subunit to function [21, 22].

Possible mutations of specific region of NAD(P)H oxidase contribute to the enhancement of the enzyme activity and subsequent increase in superoxide production (oxidative stress) [5]. Possible role for *NOX4* in nuclear DNA replication through PDI P38 interaction was reported by Modica-Napolitano & Singh (2002) [4].

PHB is a shuttle protein that transports between the subcellular compartments [12]. The data presented here concerning the role of PHB1 in survival of ovarian cancer cells was supported by explanation concerning PHB1 regulation of generation and activity of ETC production of ROS [8, 23]. PHB1 also interacts with the adaptor protein P66 ShC, a major mediator of stress induced apoptosis. This adaptor protein is activated by H_2O_2 and ROS in the mitochondria leading to apoptosis [6].

It was reported that disturbances in the subcellular localization of PHB1 provide a suitable opportunity for ovarian epithelial cells to grow through Ras-Raf-MEK/ERK activity. The phosphorylated isoform is responsible for the activation of this pathway [14]. The present results are also agree with Thuaud *et al.,* (2013) [24] who concluded that subcellular localization of PHB modulated the regulation of PHB1 by transforming growth factor beta (TGF-β) through inhibition of luteinization [24, 25]. Jiang *et al.,* (2015) [26] have reported that AKT phosphorylates PHB1 in the cytoplasm and promotes PHB1 translocation to induce bladder cancer.

In conclusion, over-expression of PHB1 and *NOX4* in malignant ovarian tissues suggest that PHB1 is associated with tumorigenesis via activation of *NOX4* enzymes with subsequent release of ROS in the cells. Thus, intense efforts should be directed at improving the understanding of the nuclear trafficking and transcriptional regulation of PHB which may have inspiring prospects in the future therapy of oxidative stress- associated diseases.

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