**Author's response to reviews**

**Title:** Antioxidant-mediated up-regulation of OGG1 via NRF2 induction is associated with inhibition of oxidative DNA damage in estrogen-induced breast cancer

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Antioxidants-mediated upregulation of OGG1 via NRF2 induction is associated with prevention of oxidative DNA damage in estrogen-induced breast cancer

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Author's response to reviewers: please see next page
Dear BMC Cancer Editorial Team

Subject: MS: 4783821308987393 “Antioxidants-mediated upregulation of OGG1 via NRF2 induction is associated with prevention of oxidative DNA damage in estrogen-induced breast cancer”.

Authors: Bhupendra Singh et al.

We are very pleased to learn that the reviewers’ comments about our manuscript were positive. Upon editors’ suggestion, we are excited to submit the revised manuscript to BMC Cancer for consideration of publication.

In this revised manuscript, we have incorporated all the suggestions of the reviewers. We therefore hope that this revised version of our manuscript will be accepted for publication in BMC Cancer.

Please note that in the revised manuscript, we have made minor changes in the title. It now reads “Antioxidant-mediated up-regulation of OGG1 via NRF2 induction is associated with inhibition of oxidative DNA damage in estrogen-induced breast cancer”. This is a slightly edited title compared to the original title “Antioxidants-mediated upregulation of OGG1 via NRF2 induction is associated with prevention of oxidative DNA damage in estrogen-induced breast cancer” and would appropriately reflect the contents of the manuscript.

Below are the itemized responses to the reviewers’ comments/concerns:

Reviewer # 1 (Dr. Gasperi-Campani)

Concern: Authors suggest that antioxidants Vit C and BHA provide protection against oxidative DNA damage and E2-induced mammary carcinogenesis, at least in part, through NRF2-mediated induction of OGG1. Did they check other possible molecular pathways/targets? It could add value to the results presented here.

Response: We have recently shown that antioxidants Vit C and BHA upregulate expression of NRF2-regulated protective antioxidant genes NAD(P)H-quinone oxidoreductase 1 (NQO1) and superoxide dismutase 3 (SOD3) in mammary tissues (Carcinogenesis 2012; 33:156-163; Carcinogenesis 2012; 33:2601-2610). We have added this information and the relevant references in the “Discussion” section of the manuscript on page 18.

Reviewer # 2 (Dr. Pellegrino Michele)

Concern: Although in the manuscript there are many syntax and typography errors, the authors elucidated in a clear and exhaustive manner, that antioxidants Vit C and BHA protect against oxidative DNA damage and E2-induced mammary carcinogenesis, at least in part, through NRF2-mediated induction of OGG1. Therefore in this paper, the data shown
seems to be convincing from a scientific point of view, since there aren’t any considerable critical elements.

Response: We thank the reviewer for pointing out syntax and typographical errors in the manuscript. We have copy edited the manuscript and carefully gone through the syntax and typographical errors as suggested by the reviewer.

Reviewer #3 (Dr. Filippo Acconcia)

Concern 1: The ability of a physiological hormone to induce DNA damage in cells, thus inducing cancer, is to me odd. This derives mainly by the fact that there is some degree of confusion in the manuscript regarding the concentration of E2 used. What is the actual concentration rats are exposed to for 240 days? Is this constant? Did the authors take into consideration the physiological states of the female rats treated (i.e., pre-menopause/post-menopause)? This point must be clarified because the effects the authors find on OGG is observed in all tissues, thus suggesting a possible aspecific effect due to the treatment itself.

Response 1: We understand and appreciate the reviewer’s concern. It is very well established that E2 induces DNA damage. We and others have earlier reported that E2-induced DNA damage is associated with breast carcinogenesis (Proc Natl Acad Sci U S A 1997, 94:10937-10942; Carcinogenesis 2000, 21:427-433; Oncogene 2007, 26:3587-3590; Carcinogenesis 2012, 33:156-163). Female, ovary-intact ACI rat model that we have used in our study is an established animal model for estrogen-induced breast cancer (Carcinogenesis 1997, 18:1595-1601; Proc Natl Acad Sci U S A 2000, 97:2779-2784; 14-19; Carcinogenesis 2006, 27(3):491-498; Toxicol Appl Pharmacol 2008, 232:78-85). It has been reported that the serum E2 levels in control, ovary-intact ACI rats oscillate between ~20 and 75 pg/ml whereas the mean level of serum E2 in E2-treated, ovary intact female ACI rats average ~100 pg/ml and remain constant during the course of the study (Mol Carcinogenesis 2002, 33:56-65). Epidemiological studies suggest that prolonged exposure of estrogens is associated with breast cancer. Literature reports suggest that increased serum estrogen levels are associated with increased risk of breast cancer (J Steroid Biochem Mol Biol. 2003, 86:477-486). Therefore, the dose of estrogen used in estrogen-induced breast cancer model is always slightly higher than that of normal circulating levels. We start treatment of female ACI rats with E2 when they are 5-6 weeks old. This age is considered puberty or early puberty stage for female rats and the duration of our study was up to 8 months which represents the fertile period of female rats. It has also been reported that female ACI rats remain in proestrus stage for the duration of study after E2 implantation (Carcinogenesis 1997, 18:1595-1601).

We have included/added this information to the “Discussion” section of the manuscript on page 15.

We have observed E2-mediated inhibition of OGG1 in all the tissues that we have tested (mammary, liver, kidney, uterus, lung and spleen) (Figure 1) compared to control rats. This suggests an estrogen receptor independent mechanism of E2-mediated inhibition of OGG1. We have also observed a similar effect in vitro in two different mammary epithelial cell lines, MCF-10A and T47D, non-tumorigenic and tumorigenic breast cell lines,
respectively. These data strongly support the hypothesis that inhibition of OGG1 is E2 specific.

**Concern 2:** In line with the above questions, cells are treated with 50nM (0.05 #M), that is a quite high E2 concentration. Moreover, in figure 3 D CHIP is performed in 10nM E2 stimulation. Why did the Authors change the E2 treatment? The Author should explain this point or eventually perform a dose-curve analysis.

**Response 2:** We again appreciate the reviewer’s concern. We agree with the reviewer that 50nM E2 is rather high E2 concentration. We chose this concentration because we as well as others have earlier shown that 50-100nM E2 induce 8-OHdG formation, an oxidative DNA damage marker (PLoS ONE 2011, 6:e25125; Carcinogenesis 2012; 33:156-163; Toxicol Appl Pharmacol. 2002;180:219-226; Oncogene. 2008;27:6376-6384). Therefore, we kept this concentration constant throughout our *in vitro* studies. However, we observed a similar inhibitory effect of lower dose of E2 (10nM) on OGG1 expression *in vitro* during our dose-curve analysis (data not shown). In the revised manuscript, we have added this statement in the “Results” section. We changed E2 concentration to 10nM for ChIP assay because we have standardized this method and have reported earlier that 10nM E2 concentration is sufficient to induce binding of NRF2 to the promoter regions of the NRF2-regulated genes (Carcinogenesis 2012; 33:156-163; Carcinogenesis 2012; 33:2601-2610).

In summary, we have addressed all the concerns of the reviewers. Therefore, we hope that the revised manuscript will be acceptable for publication in the BMC Cancer.

The revised manuscript has been uploaded on biomedcentral as advised by you.

If you have any question, please feel free to contact me at 816-235-5903 or via e-mail at bhath@umkc.edu.

Thank you.

Sincerely,

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