Author's response to reviews

Title: Natural resistance to ascorbic acid induced oxidative stress is mainly mediated by catalase activity in human cancer cells and catalase-silencing sensitizes to oxidative stress

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Author's response to reviews: see over
Cover letter with a point-by-point description of the changes made

First, we would like to thank the reviewers for their helpful comments. The revised manuscript was corrected by a native speaker.

Reviewer 1

1. More experimental data such as the effects of some other hydrogen peroxide-detoxifying enzymes are needed.
The manuscript focused data on the ability of human cancer cells to protect themselves against oxidative stress mediated by extracellular ascorbic acid-induced hydrogen production. Silenced catalase expression in the ascorbic acid-resistant cancer cell line BT-20 increased its susceptibility to oxidative stress (Fig. 6 and 7). In addition to catalase, enzymes of the peroxidase family, e.g. glutathione peroxidase, are also important for cell protection. The authors present data that the knock-down of catalase in BT-20 cells does not influence glutathione peroxidase activity (shown in additional Fig. 1). However, the uninfluenced activity of glutathione peroxidase was insufficient to protect catalase-negative BT-20 cells against ascorbic acid-mediated oxidative stress. This is in contrast to BT-20 wild type cells with uninfluenced catalase activity. In our mind these results are sufficient to hypothesize that glutathione peroxide play not a major role in protecting cancer cells against ascorbic acid-mediated oxidative stress.

2. We do not see a big difference in catalase activity from Fig. 4a and this indicated that catalase may not be the main target for ascorbic acid.
Fig 4a shows the catalase protein level normalized to β-actin and not the catalase enzymatic activity. The authors apologize for the misunderstanding Y-axis label. The authors changed the label from “Normalized catalase level” to Normalized catalase protein level”.

Since protein expression does not always correlate directly with enzymatic activity, the authors determine catalase activity (shown in Fig. 4b) which was significantly increased in the ascorbic acid-resistant cell lines SKOV-3, 23132/87 and BT-20 in comparison to the ascorbic acid non-resistant cell lines U-87 and U-251.

3. The manuscript should also provide the western blot results for some caspases.
The main message of the manuscript is that catalase is involved in the resistance of cancer cells to ascorbic acid mediated oxidative stress. Higher levels of catalase activity are found in cell lines that are resistant to oxidative stress than in more susceptible cancer cell lines. The investigation of caspase 3 and 7 activation represents only a minor part of the manuscript and the authors confirmed that ascorbic acid mediated cell death occurred by apoptosis. The activation of caspase 3 and 7 was analysed with the established luminescent assay provided by Promega. This assay is commonly used to analyse the activation of caspases (e.g. Gao P et al. J Biol Chem 2010; 285: 25570-25581)

4. In writing, detailed protocols need to be provided for all the data.
The protocols were completed. Established assays were used for analysing catalase activity and caspase activation and performed exactly according to manufacture’s instructions.
1. Expression of vitamin C transporter (SVCT) gene in each cell line should be determined because the level of SVCT is thought to be another determinant for the intra-cellular vitamin C concentration within the cell. Results published by Levine’s group show that extracellular ascorbic acid mediates cell death independent of SVCT expression. They measured cell death even in cells with little or no expression of SVCT (Chen Q et al. PNAS 2005; 102:13604-13609). In addition, they identified hydrogen peroxide as the effector species mediating ascorbic acid-induced cell death which is membrane permeable. Wu et al. (Endocrinology 2008; 149: 73-83) showed for human choriocarcinoma cells that the knock-down of SVCT2 decreased the ascorbic acid uptake by approximately 50%. In this study the authors analysed ascorbic acid in concentrations of µmol/L and not mmol/L as used in studies of Levine’s group. At this concentration ascorbic acid serves as a prooxidant that generates hydrogen peroxide. In addition, results in our manuscript show that cell lines that are extremely susceptible to the ascorbic acid mediated cytotoxic effect are sensitive to hydrogen peroxide, too. In summary, the results of Levine’s group show that intracellular ascorbic acid plays a minor role in inducing cell death at present of high levels of extracellular ascorbic acid.

2. The reason why each cell expresses different catalase gene activity should be described, including a possible regulation of catalase gene expression within the cells. It seems that many cancers demonstrate substantially lower catalase activity than normal tissues, allowing cancers to generate a moderate intracellular level of oxidative stress to aid their proliferation and survival (McCarty MF et al. Med Hypotheses 2010, 74(6):1052-1054; Szatrowski et al. Cancer Research 1991; 51: 794-798; and Sun et al. Carcinogenesis 1993; 14: 1505-1510). It is known that expression of catalase is regulated at message, protein and activity levels (Nishikawa M. Cancer Lett 2008; 266: 53-59). We could show that the tumour cell lines used in our experiments are different in their catalase activity. Szatrowski and Sun described that rapidly proliferating cells such as cancer cells generate abnormally high hydrogen peroxide levels. This and other factors increased oxidative stress during neoplastic transformation and may promote the selection of cells with modified (increased or decreased) catalase activity. The modified catalase expression in cancer cells remains puzzling but it seems that prolonged exposure to reactive oxygen species (ROS) downregulates catalase expression via hypermethylation of the catalase promoter and, in addition, transcription factors seem to be involved (Min JY et al. FEBS Lett 2010; 584: 2427-2432; Tekeuchi T et al. J Biochem 2000; 128: 1025-1031). Catalase is also down-regulated in healthy cells transformed with T-antigen of SV40 or Ras, although the underlying mechanisms of this down-regulation are still unknown (Sun Y et al. Carcinogenesis 1993; 14: 1505-1510; Hoffschir F et al. Carcinogenesis 1993; 14: 1569-1572). Interestingly, it also has been observed that catalase levels are modified in cancer cell lines resistant to some chemotherapeutic agents or hydrogen peroxide (Xu H et al. Biophys Res Commun 2005; 328: 618-622; Akman SA et al. Cancer Res 1990; 50: 1397-1402). In summary, catalase expression is regulated in a wide array of cellular processes. The information in this paragraph was included in the discussion.

3. Arrows in Fig. 3 should be omitted.
   We omitted arrows.

4. Presentation of p values is not correct in Fig. 4. It should be presented by “less than (<)” not equal (=) as in the legend.
We changed presentation of p value. We thank the reviewer for drawing the author’s attention to this.

**Reviewer 3**

This reviewer has no questions.