Author's response to reviews

Title: Modulation of TRAIL resistance in colon carcinoma cells: Different contribution of DR4 and DR5

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Author's response to reviews: see over
Dear Editor:

Thank you for considering our manuscript entitled ‘Modulation of TRAIL resistance in colon carcinoma cells: Different contributions of DR4 and DR5’ for publication in *BMC Cancer*.

Based on the helpful suggestions of the reviewer, we have changed our manuscript, and consider the final version to be much improved. The manuscript has notably been proof-read by a native English speaker and we consider it has greatly benefited in readability. The reviewer’s comments and changes made are outlined below. We also brought some improvements to the manuscript, which we report at the end of this letter.

I apologize for the delay in resubmitting this manuscript. We hope that you will be able to accept our revised manuscript for publication in your journal.

Yours sincerely,

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Response to editor and reviewer:

All of the suggestions made by the editor and reviewer have been listed below. We have addressed the comments on the manuscript on a point-by-point basis.

Editor

Quality of written English: Needs some language corrections before being published.

We thank the editor for this helpful remark. We had the manuscript edited by a native English speaker, and consider that the readability of the manuscript has greatly improved.

Reviewer #1

We thank reviewer #1 for his clear and interesting comments.

a/ On pg. 10 the authors claim that possible mutation in DR5 extracellular domain could affect TRAIL-DR5 interaction and thus they should address this possibility by sequencing DR5 in SW948 cells.

In Figure 1, we show that TRAIL effectively binds to DR5 in both SW948 and SW948-TR. Furthermore, we show that the ratio between DR4 and DR5 and amounts of proteins recruited at the DISC, i.e. DR4 and DR5, the adaptor protein FADD are similar in SW948 and SW948-TR, except for the lower levels of caspase 8 in the latter (Figure 1B). Since heterotrimerization of DR4 with DR5 has not been found in any report to our knowledge, these results indicate that rhTRAIL binds both to DR4 and DR5. In addition, cleaved caspase 8 was detected in the rhTRAIL-induced DISC demonstrating functionality of the DISC. Functionality of DR5 was also demonstrated in SW948-TR, the isogenic subline of SW948, with DISC IP using agonistic DR5 antibodies, since cleaved caspase 8 was detected in the agonistic DR5 antibody-induced DISC at a similar level as found with agonistic DR4 antibody. Short term incubation with the protein synthesis inhibitor CHX that resulted in FLIP downregulation ((van Geelen et al., Int J Oncol 2010; 37: 1031-1041) could maximize sensitivity of SW948 to agonistic DR5 antibody (Figure 3B) and sensitize SW948-TR cells to rhTRAIL and agonistic anti-DR5 antibody (Figure 2C and 3B). Taken together, our results demonstrate the absence of mutations and post-translational modifications in DR5 that might have prevented the binding of rhTRAIL or agonistic antibodies to DR5 and the subsequent formation of the DISC. Downstream events preventing efficient caspase 8 cleavage seem therefore more likely.

For clarity, we edited the sentence (p10, line 17) to “A competition experiment was performed in SW948 to look whether rhTRAIL binding to DR4 or DR5 was affected”, thus removing the mention of possible mutations. We also added the following sentence in the discussion: “This strongly suggests that DR5 is functional in both cell lines, as also indicated by rhTRAIL binding and comparable DISC formation between DR4 and DR5” (p16, line 7).
b/ The authors claim that at the DISC level DR4 and DR5 signaling in ETR1- vs TR2J-treated cells is comparable (Fig. 4A), i.e. DISC assembly and processing of caspase-8 proceeds with similar efficacy. However, they just analyzed an early time point – 15 min, and it is rather possible that the differences in ETR1 vs. TR2J-signaling could unveil at later time point. Thus, they should examine DISC composition e.g. 45 min after adding the agonistic antibodies. This might also explain significant differences in caspase-8 processing observed in the total lysate (Fig. 4B)

We were interested in the initial DISC formation, prior to events such as receptor internalization and signal amplification. Our experiment shows that after 15 minutes the DR4-DISC and the DR5-DISC is efficiently induced using the DR4 or DR5 selective agonistic antibodies. In a recent publication, we showed that the turn-over rate of FLIP and caspase-8 at the DR5-DISC and not the delayed recruitment of caspase 8 to this receptor determines DR5-mediated apoptosis signaling (Pennarun et al, Anal. Cell Pathol./Cell. Oncol.,2010 Oct 26. [Epub ahead of print]). Thus, DISC analysis at early time-points (15 minutes) constitutes a valid indication of efficient DISC recruitment (as also seen in Figure 1B). We added the sentence "This is consistent with our recent findings that caspase 8 cleavage at the level of DR5-DISC in SW948 is due to lower turn-over of DISC components rather than to decreased DISC formation (Pennarun et al, Anal. Cell Pathol./Cell. Oncol.,2010 Oct 26. [Epub ahead of print])]." (p16, line 15).

c/ According the authors the acquired TRAIL resistance of SW948-TR cells is caused by downregulation of caspase-8 expression in these cells (Fig. 1C), which is being reversed by IFN-γ treatment. But IFN-γ could have additional effects on these cells (e.g. by downregulating FLIP expression etc.). Thus it would be instrumental to reconstitute (or better to say increase to the level in SW948 cells) pro-caspase-8 levels in SW948-TR and analyze an effect on TRAIL-induced apoptosis.

We agree with the reviewer’s comment that this experiment does not allow to conclude that lower caspase 8 levels cause TRAIL resistance in SW948-TR. However, in a recent publication describing how the SW948-TR cell line was generated, we demonstrated the importance of (high) caspase 8 levels for TRAIL sensitivity in the parental cell line SW948 (van Geelen et al., Int J Oncol 2010; 37: 1031-1041). We also showed in this publication that the low levels of caspase 8 in SW948-TR, while FLIP levels remained unchanged, caused TRAIL resistance. Downregulation of FLIP enhanced capasase-8 cleavage and restored TRAIL sensitivity in SW948-TR. In addition, downregulation of procaspase 8 levels in the TRAIL sensitive SW948 to a similar level as found in SW948-TR was sufficient to induce TRAIL resistance in SW948. We therefore referred to these previous findings in the result section: “Reduced caspase 8 expresion levels were detected in SW948-TR as compared with SW948 (figure 1C), which is causative for the observed rhTRAIL resistance in these cells (van Geelen et al., Int J Oncol 2010; 37: 1031-1041).” (p13, line 19) and in the discussion “Downregulation of caspase 8 levels in SW948 – that is, to a level comparable to that which is normally observed in SW948-TR cells – was sufficient to induce TRAIL resistance, indicating the importance of the caspase-8/ c-FLIP ratio in these cells (van Geelen et al., Int J Oncol 2010; 37: 1031-1041).” (p17, line 7)
We have added a Western-blot analysis demonstrating that IFN-γ does not reduce the expression of c-FLIP. The Western blot has been included in the new draft of the manuscript (Supplementary Figure 1). Accordingly, the following sentences were added in the result section: “We also assessed whether IFN-γ modulated c-FLIP levels. Changes in c-FLIP expression could have affected caspase 8 cleavage in response to the various pro-apoptotic TRAIL receptor ligands (Supplementary Figure 1). Western-blot analysis showed that IFN-γ induced some cleavage of c-FLIP. However, IFN-γ did not change basal c-FLIP levels.” (p14, line 21)

d/ The authors mention that DEAD-box helicase DDX3 could negatively influence DR5-triggered apoptotic signaling via its interaction with the intracellular part of DR5. Thus it might be worthy to examine DDX3 association with TR2J-DR5 DISC.

It was demonstrated that DDX3 acts upstream of the DISC by preventing FADD recruitment. We found that FADD was efficiently recruited to DR5 in our cell line, as shown in our DISC IPs. In addition, a recent publication showed that DDX3 was not specific for TRAIL-R2 but also binds to TRAIL-R1, thus similarly blocking apoptosis signaling via both receptors (Sun et al, Cell Death and Differentiation, 2008, 15: 1887-1900). We therefore removed from the discussion the section commenting on the specificity of DDX3 for DR5 (p17, line 21). The role of DDX3 in TRAIL resistance in general, however, may still be interesting for further study. Several mechanisms have been shown to differentially regulate DR4- and DR5-mediated apoptosis (Pennarun et al., BBA-Rev on Cancer 2010, 1805: 123-140). We are currently following some of these other leads.

Minor textual adjustments and improvements throughout the text

Antibody names

For the purpose of readability, the generic names of the pro-apoptotic antibodies from Human Genome Science were replaced throughout the text by the terms “DR4 agonistic antibody” and “DR5 agonistic antibody”. The generic names of these antibodies have been retained in the M&M section.

Slight upregulation of DR5 following IFN-γ treatment

IFN-γ treatment resulted in a slightly reduced DR5 surface expression level, which could partly explain why, despite the increase in caspase 8 levels, DR5-mediated sensitivity did not increase. We therefore added the sentence “IFN-γ treatment slightly downregulated surface DR5 levels but not DR4 levels” (p13, line 23)