Author's response to reviews

Title: MDM2 SNP309 is associated with high grade node positive breast tumours and is in linkage disequilibrium with a novel MDM2 intron 1 polymorphism

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Author's response to reviews: see over
MDM2 SNP309 is associated with high grade node positive breast tumours and is in linkage disequilibrium with a novel MDM2 intron 1 polymorphism

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Dear Dr Cassady-Cain

Many thanks for your e-mail of 7th July 2008 with the referees’ comments to our manuscript (4529573891938054). We have considered each of the helpful points raised as outlined below and where appropriate have amended the manuscript.

One of the concerns that Referee 3 raised, and was highlighted by yourself, was the fact that our study could be underpowered. We have therefore presented power calculations for our study in this letter of response using the method (and accompanying software) published by Menashe I, Rosenberg P, Chen B: PGA: power calculator for case-control genetic association analyses. BMC Genetics 2008, 9(1):36.

In relation to Table 1, based on a co-dominant inheritance pattern, a sample size of 300 cases, p of 0.05 and 80% power, the minimal detectable relative risks in our study are: 1.65 for the G/G genotype (prevalence of 11%) and 1.4 for the T/G genotype (prevalence 42%). Had we studied 1000 cases then these figures would only have fallen to 1.37 and 1.25 respectively. This indicates that we did have sufficient power to detect a doubling or halving of risk, but we cannot exclude the possibility that SNP309 increases or decreases the risk by a much more modest level of 10%. However, our study is of comparable size to many other published studies and indeed our cohort sizes are considerably larger than those reported in the seminal paper published by Bond et al, Cell 2004,119:591 (Control cohort-50, Li-Fraumeni cancers-66, Breast cancer Li-Fraumeni patients-13). Furthermore, a meta-analysis of all the published literature on breast cancer and SNP309 to 2007 (11 studies) looking at 5737 cases and 6703 controls also revealed no association with breast cancer risk (OR=0.97, 95%CI=0.87-1.08) (Wilkening S, Bermejo JL, Hemminki K: MDM2 SNP309 and Cancer Risk: A Combined Analysis. Carcinogenesis, 2007, 28(11):2262). The upper bound of this confidence interval (1.08) is within our calculated confidence intervals and therefore our results are also consistent with this pooled analysis.

In relation to Table 2, multiple testing could be an issue. However, we have provided Odds Ratios and confidence intervals so that readers can make up their own minds as to what they might consider “significant” (or not). Alternatively, we could apply a simple Bonferroni correction (multiply each p value in each 3-way comparison by 3). If we do this then the comparisons of NPI G/G vs. T/T and Tumour Grade G/G vs. T/T become non-significant. However, the hazard ratios suggest that these
differences are genuine and the non-significance of the adjusted p values merely reflects the small number of patients with G/G (and consequent lack of statistical power).

In conclusion, although not powered to detect a 10% difference in risk ratio of breast cancer, we feel that our study is adequately powered and is in common with all the other studies published so far. Similarly, in keeping with the majority of publications, we did not find evidence of a link between breast cancer risk and SNP309, however this was not the main aim of our manuscript. We instead focussed on our findings of an association between SNP309 and key adverse prognostic factors and also demonstrated that SNP309 is in linkage disequilibrium with SNP285, a novel MDM2 intron 1 polymorphism. This in turn may affect the penetrance of SNP309 and thus account for some of the discrepancies in the literature. As highlighted in my original letter to you accurate sequencing of this region is of particular importance as within the materials and methods section of a recent publication (Atwal GS, et al., Proc Natl Acad Sci USA 2007;104:4524) a statement was made to indicate that in a previous publication (Harris SL, et al., Proc Natl Acad Sci USA 2005;102:16297), 32 out of 113 samples had been incorrectly genotyped with respect to SNP309, and 22 out of 113 samples were duplicates. This therefore meant that the original conclusion that SNP309 was associated with a low apoptotic frequency is no longer valid. While this is stated within the materials and methods section of the second paper (Attwal et al., 2007), and no formal retraction has been published, it would also imply that the influence of SNP309 is much more complex than originally hypothesised. Potentially SNP285 could play such a role and therefore researchers should be made aware of our findings.

I have then addressed each of the reviewer’s individual comments below:

Referee 1 (Diana Eccles)

Major compulsory revisions
1) Although the authors observed an association of the 309G/G genotype with higher tumour grade, the numbers of patients with this genotype are rather small. A major difficulty with the data is that it is difficult to work out a biological reason why one copy of the SNP (309G/T) should have an opposite effect (low grade) whereas 309G/G leads to the highest average grade – one would expect if the SNP was influencing grade that one would see a progression from low to high grade through the addition of the G allele ie T/T lowest through to G/G highest. The small numbers and the lack of progression suggest this could well be a type 1 error (very common in small association studies) and this is not adequately explored in the discussion. The correlation of higher grade (particularly in ER positive tumours which most of them were) with LN positive disease is not surprising.

These thoughtful comments have led us to add comments in the discussion emphasising the potential associations we observed more diplomatically (Page 14, Paragraph 6, Lines 1-4). As the referee correctly points out, we do provide evidence of an association which will require further testing with future cohorts. However, we would add that allele dosage effects are not always consistent and we feel it important to report the observed associations rather than be censorious in our reporting. A possible explanation for the lack of progression from low to high grade through the addition of the G allele has been added to the discussion and also covered in point 4 below (Page 15, Paragraph 7).

2) A step further would be to ask if the SNP is having any influence on actual prognosis and is the effect independent of or entirely because of the proposed influence on LN status and grade – this would require a survival analysis. The very low frequency of the additional SNPs identified in MDM2 prohibits any really useful tests of association in such small numbers of cases and by the time it gets down to 3 double homozygotes at 309G/G plus 285C/C it becomes somewhat statistically irrelevant. The small numbers at this stage are acknowledged.
We agree with the referee that a survival analysis is desirable: however, because of the low numbers of events observed, no statistical significant differences were seen and therefore we omitted this analysis from our manuscript. With longer (5-10 year) follow up, this may be possible to analyse.

3) The discussion is unnecessarily long, paragraph 2 is largely repetition, the discussion in general is not very clear. The authors state that "Taken together the data [does this mean the data in the paper plus the literature?] "suggest that different grades of cancer in fact represent different cancer subtypes”, which theory is further expanded in the final sentence in the first paragraph of page 15 into an incomprehensible extrapolation.

Paragraph 2 of the discussion has been shortened to avoid repetition of the results section. This is useful guidance; paragraphs 7, 8 & 9 have been condensed into a single paragraph clarifying the discussion and incorporating point 4 below.

4) The observation of an association between the 309G/G genotype, grade and lymph node status is certainly of interest and worth exploring further. However given the nature of this type of study it is very important not to over interpret the data. It would be helpful in presenting the data to try and come to some sort of tenable hypothesis about how this SNP might be influencing grade given that it does not appear to be an additive effect with each G allele contributing more to grade.

As in referee’s point 1, the discussion has been amended highlighting the preliminary nature of our findings.

One potential explanation of why each G allele may not appear to act additively, is to hypothesise that the G allele binds one factor (X) and the T allele binds a different factor (Y). The heterozygotes would therefore bind both factors X and Y and it is the levels of these “factors” that determine the overall levels of MDM2 produced. In the last paragraph of the discussion potential transcription factors known to bind around this SNP region are mentioned and thus the interplay of these factors may determine MDM2 levels. One key factor highlighted is p53, which is known to show haploinsufficiency, and therefore MDM2 genotype combined with tumour p53 functionality status may in fact be determining tumour phenotype. This has been added to the discussion (Page 15, Paragraph 7).

**Minor Essential Revisions**

1) Tumour grading is subjective and specialist breast pathologists tend to grade higher than general pathologists so some description of how tumours were graded, and who by (one read or two, consensus etc) would be important given the conclusions that are reached.

Tumour grading was carried out by a single specialist breast pathologist (CAP) and graded as defined by the NHS Breast Screening Programme guidelines. This information had been added in the Materials and Methods section (Page5, Line 6 & Ref 17).

2) ER, PgR scored using IHC, for HER2 the authors state that samples were scored by IHC and FISH – did all samples have both? HER2 scored 2+ on IHC is not usually considered positive without FISH confirmation – the sentence need to be reworded to clarify that.

HER-2 samples were evaluated by IHC and samples scoring 2+ by IHC were then subjected to FISH. Samples scoring IHC 3+ or IHC 2+/FISH +ve were considered to be HER-2 positive. We agree that this was not clear in the original text and have amended this accordingly (Materials & Methods, Page 5, Line 11).
3) The number of controls should be stated in the methods section with mean age and range.

The number of controls with the mean age and range is now stated in the Materials and Methods section (Page 5, Line 12).

4) Selection of controls is always a difficult issue and there is no real consensus on the ideal control group for association studies but the composition of the control group should be clarified – particularly age. If controls are younger than cases they may become cases in future. If older than cases they may be “hypernormal” (ie old age and no cancer) controls. The former could potentially reduce the observation of any difference between cases and controls and the latter could increase the chance of observing an association.

The mean age of the control cohort was slightly younger by 6.3 years, the range of ages was, however, larger. By adding information on the mean age and range this is now addressed and stated in the text (Materials & Methods, Page 5, Line 12).

5) Ethnic similarity can be confirmed by genotyping and for an association study would be important to establish or at least make some comment about what is known about the ethnic variation in the population sampled. In small numbers as in this study, an admixture of ethnic minorities in one group or another could easily bias the results especially as the authors comment on the wide variation of 309 genotypes in other ethnic groups and differences in tumour type and prognosis are well described between different ethnic groups, especially black african women.

Both the control and breast cancer cohorts were from the Tayside region, and consisted of only Caucasian individuals (which is already stated in the Materials and Methods section - Page 5, Line 13) so this would not have an impact on our study.

Referee 2 (Sharon Pine)

1. How were the controls for this study recruited? Were there any differences in demographic variables between those who agreed and disagreed to participate?

Control samples were randomly selected from a larger Tayside control cohort of anonymised samples previously used in population association studies and thus differences in the demographics of those who agreed and disagreed to participate is not pertinent to the present study.

2. A table comparing the demographics of the cases versus controls is needed, including age, menopausal status, family history of breast cancer, household income, and education level.

As the control samples were of similar age, and were all of Caucasian origin to the cases, we have elected not to include an additional table. Information on menopausal status and family history of breast cancer were not included as controls were not compared to breast cancer cases with respect to these parameters. Household income and educational level fall out-with the remit of this study.

3. Please indicate the referent group for each analysis shown in Table 2.

Each genotype was compared to all the other samples for this logistic regression analysis and this has now been added to the figure legend for Table 2 (Page 23) to make this clear.

4. Is there an association between SNP309 and tumor histology?
Assuming this refers to histological tumour type, 85% of invasive tumours were ductal and only 15% of other types (lobular and other special subtypes). Therefore the numbers within each subcategory of non-ductal cancers would be too small to warrant testing for an association. The association with tumour grade was examined and is presented as a significant association (Results, Page 8, Line 2).

5. The 20 cases with DCIS should not be included in the risk assessments or in the comparisons to clinical characteristics in Table 2, because DCIS patients may not represent a true breast cancer population. Alternatively, stratify risk analysis by DCIS versus invasive breast cancer, and/or justify why DCIS patients are included in analyses. The lack of the 309G/G genotype among DCIS patient is of merit and supports its association with high tumor grade and should still be included.

Ductal carcinoma in situ (DCIS), although representing non-invasive breast cancers, are classically picked up by the breast cancer screening program and are treated as a subset of breast cancers. A high proportion go on to develop invasive breast cancer if left untreated. We therefore felt that they should be included in the analyses. Subdividing the analysis into invasive and non-invasive cancers would be of merit, however, given the small numbers of non-invasive cancers (6.7%) this would not be likely to provide a statistically significant association. Moreover, as the reviewer’s comments, stating that we did not observe the 309G/G genotype in the DCIS patient group is potentially interesting, rather argues that DCIS should be included in the analysis.

6. On page 10, in the “Additional MDM2 intron 1 polymorphisms” section, SNP285C was shown to be associated with breast cancer risk. Were the male controls included in the analysis? Please show the N values for each allele in the cases and controls.

Both male and female controls were included due to the small numbers within each of these rare genotypes, but distributions between the sexes showed no variation. This has now been added to the legend of Table 3 (Page 23) for clarity.

The N values for each allele in the cases and controls are already present in Table 3 – 3rd and 7th columns

7. The discussion on page 11 states that 275 cancer free controls were used in the study to correlate MDM2 SNPs with age of diagnosis, pathological variables, and clinical outcome. However, this is not the case. First, the controls were not used for correlating the SNPs with age at diagnosis, or pathological variables. Second, clinical outcome was not reported.

This helpful comment was due to our grammatical error and has now been changed to read: The intron 1 region of MDM2 was examined in a Scottish population of 299 breast cancer patients and 275 cancer free controls to establish any associations between MDM2 SNPs and breast cancer. In the breast cancer cohort MDM2 SNP309 was also analysed with respect to age at cancer diagnosis and pathological variables. (Discussion – Page 11, Lines 1-4). The phrase clinical outcome has been removed.

8. Please state the limitations of the study in the discussion, in particular, that the numbers of cases and controls are low (especially in association between the SNP309 homozygous variant group and prognostic variables) and are not matched on demographic features.

These points have now been highlighted in the discussion emphasising the preliminary nature of this study and how the findings are suggestive of links and associations rather than being definitive categorical links.
Referee 3 (Alfons Meindl)

Major concern:
1. The study is underpowered and the findings might be replicated in larger sample sets. The authors should try to validate their findings in more tumor samples with grade 3 and in patients with a lymph node negative diagnosis.

This point is discussed in the opening paragraphs of this letter fully. We used an unbiased approach to examine 299 breast cancers that we had full clinical data for and suitable DNA to PCR amplify and sequence. Our original cohort was larger (>400 samples) but due to failure in DNA quality or lack of clinical information these samples were not included in the analyses. The referee’s suggestion of examining more grade 3 samples and more lymph node negative patients would make an interesting future programme of work in a much larger cohort study.

Discretionary revisions:
1. I would focus the manuscript on the main message and keep the informations about the other issues much shorter.

This helpful comment has been taken on board and the discussion has been amended accordingly. (Discussion - Paragraph 2 has been condensed and Paragraphs 7, 8 & 9 have also been condensed into a single paragraph).

We trust that all the points raised by the referees have be answered adequately and hope that they will now satisfy any concerns you may have over publishing this manuscript. However, if you require further clarification on any additional points we would be happy to provide you with this information.

I look forward to hearing from you.

Yours sincerely

Fiona E M Paulin