Author's response to reviews

Title: High-resolution array CGH clarifies events occurring on 8p in carcinogenesis.

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Author's response to reviews: see over
We thank the reviewers for their comments that our work was ‘well performed and the data are carefully analyzed’ and ‘very elegantly done’. We appreciate the reviewers’ input, particularly that of Dr Hoglund whose suggestions add substantially to the paper. We agree with almost all their points and have been able to accommodate almost all of them in the revision.

Reviewer 1: Mattias Hoglund (corrections highlighted in blue in revised text)

Major Compulsory Revisions

1. Page 7 and page 9, the Chi-squared test. The authors use a chi squared test to investigate if rearrangements on 8p occurred without selection. A chi-squared test is not a test for selection but a test for deviations from random events. Hence, the test shows that the breaks on 8p are non-random but it does not give an explanation for this. The authors interpret the data as the presence of a selective pressure. An alternative, and a just as valid explanation with the given data, is the presence of a break prone region in one of the bands. My suggestion is that the authors include this as an alternative explanation.

We agree that there are alternative explanations for the non-random distribution of breaks suggested by the chi-squared test. We have clarified that the test null hypothesis is a random distribution of breaks owing to ‘the absence of any selection or pre-disposition to breakage at certain sites’ (page 7) and that the interpretation of this result is that ‘rearrangements of 8p are under selective pressure or that breaks occur non-randomly due to breakage-prone regions’ (page 9).

2. Page 10. A novel amplicon in 8p21.3. Here the authors identify a recurring small amplicon in 8p21. The authors then perform FISH analysis to study the genomic organization of the amplicons and finally investigate the frequency of this amplification in primary tumours by performing FISH on a TMA containing 98 tumours. This is very elegantly done. In the discussion part (page 15) most of the genes in the amplification are mentioned and arguments, based on inferences derived from bioinformatic and data base data, for them being possible candidate genes are given. Here the link between increased copy numbers and increased expression is missing. Do the mentioned genes in fact show increased expression when amplified? Just as mutation with concomitant LOH is a sign for a tumour suppressor gene, increased copy numbers linked to increased gene expression is sign for a dominantly acting oncogene. Obviously the authors have the technique and material to investigate gene expression levels in both tumours and normal material (see analysis of TUSC3). As the investigation is very much focused on candidate genes, information on the link between increased gene copies and expression for the mentioned genes would greatly improve and make the manuscript much more convincing. In fact I think this type of data is necessary for the conclusions the authors would like to arrive at i.e. pinpointing candidate genes.
We agree that this type of analysis would add to the work and we have recently completely further experiments, which we have now included in the text, as follows, and illustrated in Figure 2.

**Results (p11-12)**

‘Expression of the genes within the amplicon, GFRA2, XPO7, NPM2, FGF17 and EPB49, was analysed to see if amplification had an effect on expression levels. Quantitative RT-PCR was performed on cDNA from the two cell lines that had amplification, and a further four cell lines without amplification in this region (Figure 2f). DOK2 was excluded from quantitative expression studies as expression was not detectable by conventional PCR in normal breast line HB4a, BT-20 or MDA-MB-134 (results not shown). MDA-MB-134 showed over-expression of GFRA2, XPO7, NPM2 and FGF17 compared to HB4a but 0.5-fold expression of EPB49, consistent with the edge of the amplicon falling within this gene (Figure 2f). BT-20 showed marginal, up to 3-fold, over-expression of XPO7 and EPB49. Interestingly, one cell line, DU4475, showed huge over-expression of NPM2 and FGF17 in the absence of amplification (Figure 2f).

Since NPM2 and FGF17 were over-expressed in two cancer cell lines, expression levels were analysed in 61 primary tumours, from the primary breast tumour series analysed by FISH, for which cDNA was available (Figures 2g and 2h). However, cDNA was unavailable for the tumour that showed 8p21.3 amplification by FISH. For both genes, cDNA made from five commercial normal breast RNAs showed extreme variation – over 100-fold difference – in expression across the samples, highlighting the problem of identifying suitable normal controls for primary tumour samples. The variation between commercial normal samples may reflect differences in how they are obtained, for example if RNA is extracted from tissue obtained after reduction mammoplasty it may be more representative of adipocytes than breast epithelium. These technical concerns may limit the utility of commercially available normal RNA as a reference. Purified breast luminal and basal cells [36] were included as further controls and tended to be more consistent in their expression levels.

None of the tumours expressed FGF17 above the range of the control groups (Figure 2g). Three tumours expressed NPM2 at a higher level than any of the commercial controls, although at a lower level than the normal luminal and basal cells (Figure 2h). Although tumours do not express either gene outside the range of normal controls, there are four outliers/probable outliers for each gene, which have expression at significantly higher levels than the rest of the tumours. Due to the variability of the normal controls the most significant observation may be that there were outliers over-expressing both genes within the tumour group (Figures 2g and 2h).’

**Discussion (p17)**

‘Of these genes FGF17 and NPM2 showed over-expression in two cell lines, one with (MDA-MB-134), and one without (DU4475), amplification. However, neither was over-expressed in the second cell line with amplification in this region (BT-20).’

3. Pages 11-13, 8p22 rearrangements. Here the authors base their reasoning on five cell lines. They complement their array analysis with single copy FISH and finally conclude that the most likely target for 8p22 deletions is TUSC3, which makes it a candidate tumour suppressor gene. To further test this hypothesis the
authors analyse the TUSC3 expression in 61 primary breast tumours and find a generally lower expression of this gene in the tumours. This is then used as an argument for TUSC3 being a TSG. I do not find this very convincing. The standard way to identify a TSG is to perform mutation analyses. Furthermore, given the fact that tumours show very different expression profiles compared to normal samples and that many genes are affected, one may ask what the probability is to find a gene that show reduced expression (below the range in normal samples) in 21% and absence of expression in 10% of the tumours when compared with normal samples. I guess quite many.

We accept the criticisms of our arguments raised here. Our initial reasoning was that, if we take the minimum region of loss in 8p22 as indication that one of the three genes in this region is a TSG (as opposed to there being a fragile site here – we address this point in response to later requested revisions), then loss of TUSC3 expression in primary tumours becomes a reason to favour it as a candidate only over the remaining two genes in this region. The probability of any given gene showing reduced expression in tumour samples when compared with normal will be quite high if we study every gene in the genome but as we are only considering three genes the theoretical false discovery rate will be quite low. The reason for carrying out expression analysis (apart from the relative difficulty of mutation screening which is discussed below in response to comment 4) was that this would identify reduced TUSC3 expression resulting from methylation (which has been previously reported at this locus) as well as by deletion and mutation. This therefore seemed to be the most efficient approach in terms of obtaining results to either support or rule out TUSC3 as a candidate, rather than definitively identifying it as a TSG. However, we entirely accept that this selective approach cannot constitute proof and we have therefore re-phrased our discussions of TUSC3 to reflect the fact that we merely consider it to be merely the best candidate out of the three genes in this region, rather than a bone fide TSG (See pages 14, 17-18). Please also see our response to this reviewer’s concluding comments for clarification on our aims in this paper.

Furthermore, the argument only makes sense if the loss of expression is associated with genomic alterations, which was the method used to identify TUSC3 in the first place.

This is a valid point and we have now carried out quantitative expression analysis for TUSC3 and DLC1 in cell lines with and without rearrangement of 8p22 (SGCZ was excluded as PCR failed to detect any transcripts in normal or cancer cell lines with and without rearrangements in the region as described in the manuscript). We have included this in the text as follows and illustrated it in Figure 3.

Results (p14)

‘DLC1 expression was decreased in 62 % (8/13) cell lines analysed by quantitative PCR, including four lines without detectable genomic changes in this region (Figure 3c). In contrast, TUSC3 was affected by two small deletions that did not include any other gene. TUSC3 showed decreased expression in these two lines with deletions but not in any other line (Figure 3c). Anecdotally, TUSC3 also showed decreased expression in a panel of 61 primary breast tumours (Figure 3d). This identified six tumours that had completely lost expression of TUSC3, and a further thirteen that expressed it at a lower level than the range observed in normal breast luminal and basal cells.’

Discussion (p17-18)
‘If a tumour suppressor gene is present in this region, then TUSC3 (15.6 Mb), previously suggested as a potential target in this region [4] [51] [52], was the strongest candidate target of the rearrangements of 8p22 in this study. Two cell lines have deletions that solely affect TUSC3 and result in decreased expression and expression was lost or decreased in 31% of primary breast tumours. However, the presence of several breakpoints in this region that do not appear to have an effect at the gene level may also suggest that this site is prone to breakage.’

In addition, the treatment of TUSC3 is at odds with how the authors reason regarding CLN8 in the next section. Here CLN8 is excluded as a candidate gene because it shows reduced expression in the majority of the cell lines, in addition the hypothesis that ARHGEF10 is a TSG is tested by mutation analysis (!). My suggestion is that the authors perform mutation analyses of TUSC3 as this is the standard analysis for candidate tumour suppressor genes (and is used in the subsequent section). The expression data would still be valuable but as a lot weaker argument.

We apologise for this point, which we believe is due to a lack of clarity in the English that we used. We have now re-phrased this sentence to read: ‘Expression data for multiple cancer cell lines (not shown) suggested ARHGEF10 as a more likely target gene than CLN8, since ARHGEF10 expression was decreased by more than 50% in 83% (15/18) of lines tested.’ (p15)

We hope that this clarifies that it was ARHGEF10 that showed reduced expression in the majority of cell lines and therefore we considered this to be the best candidate target gene and hence we performed mutation analysis for this gene.

We have decreased the importance placed on the expression analysis of TUSC3 as described by the revised sections above.

4. Page 13, loss of 8p23.3. It is claimed that CLN8 expression was decreased or lost in the majority of the cell lines. The authors could maybe be more specific here giving e.g., percentages and thresholds. Why was ARHGEF10 sequenced in only DU4475 and not in all cell lines? The strongest argument for ARHGEF10 to be a TSG would be if the gene is only mutated when one of the alleles is lost. To be able to claim that ARHGEF10 is a TSG the authors have to perform mutation analysis in a larger cohort in both cell lines and in primary tumours.

As clarified in the previous section it was ARHGEF10 expression that was decreased or lost and we have now added the threshold and percentage as shown above. (p15) ARHGEF10 was sequenced only in DU4475 because this was the only cell line with a heterozygous deletion and therefore the cell line which, according to classical models of TSG inactivation, would be most likely to have an inactivating mutation in the remaining copy. We accept the point that since the mutation causes a substitution we cannot actually deduce that it is an inactivating mutation rather than an oncogenic one. However, since this is a region of loss including homozygous deletion in other lines we were only testing the hypothesis that ARHGEF10 is a tumour suppressor gene and our findings do not contradict this hypothesis. ARHGEF10’s function as an activator of RhoB would also support a tumour suppressor rather than oncogenic role for this gene. We entirely agree that finding multiple mutations in primary tumour material would be definitive in identifying ARHGEF10 as a tumour suppressor and that following our analysis it remains
only a candidate gene. However, there are experimental obstacles to further mutation screening. Firstly, the quantity of primary tumour material we have available is limiting. Secondly, ARHGEF10 is a large gene requiring 28 PCRs and therefore a minimum of 56 sequencing reactions to sequence it in each sample. To do this on a number of primary samples is not trivial in terms of cost, time or the quantity of tumour DNA needed. Thirdly, identifying appropriate tumour samples to screen is not straightforward. In the absence of copy number data we cannot identify samples that have heterozygous deletions and are therefore most likely to contain point mutations. The low cellularity of breast tumours and the likelihood of heterozygosity at the locus mean that even at the site of a point mutation the normal sequence will be dominant on a sequencing trace and therefore mutations are hard to detect. The large-scale sequencing effort detailed in Wood et al. 2007 and Sjoblom et al. 2006 identified two heterozygous missense mutations in ARHGEF10, strengthening its status as a candidate cancer gene. This point is included in our discussion (p18).

5. Pages 17-18, a parsimonious model. Here the authors sum up their findings into a model that has the aim to explain the over all confused picture of events occurring on 8p (page 4). I have no problems with the model but I do find it too focused on selection of (candidate) tumour suppressor genes. One could in fact apply Occam’s razor and exclude selection for tumour suppressor genes from the model and instead introduce unstable genomic regions with a high probability to break. Given some level of genomic instability, either ongoing or transient, such regions would be detected and show up as recurrent changes. Hence, I would prefer the term “candidate regions”, also used by the authors on page 18, instead of TSG in the model.

This is an interesting point for discussion. We have now included a paragraph (p20) discussing the presence of fragile sites as an alternative hypothesis for the pattern of rearrangements seen on 8p although we still find selective pressure due to the presence of cancer genes a more convincing argument. We suggest that the lack of any reciprocal rearrangements – where distal 8p is retained e.g translocated to another chromosome – favours the theory that it is loss of sequence that is relevant rather than just a breakage event at a particular site. However, it could be counter-argued that breakage at a fragile site could always be followed by loss of the chromosome segment that does not contain the centromere. The potential existence of fragile sites on 8p has been evaluated before and we have now included the following references to this in our discussion ‘Although we propose that the driving force behind 8p rearrangements is the presence of cancer genes, the same pattern of rearrangements might be observed if the driving force was merely the presence of fragile sites on 8p. For example, it has previously been hypothesised that the breaks at 8p12 with proximal amplification could be due to the presence of a fragile site that initiates breakage-fusion-bridge cycles [61] although no fragile site has been shown to exist on 8p [62] and 8p12 breaks frequently occur without accompanying amplification.’

In addition we have changed several words in our model to emphasise that it is only one of the possible explanations and that while we propose candidate genes in several regions we do not consider that they have been proved to be TSGs (p18-19).
6. Figure 1. The colour codes in figure 1 are confusing. The consensus colour coding is red for “high” or “increased” either as in “highly expressed” or as in increased copy number and green for “low” or “decreased”. It would be an advantage if the authors could change the colour coding. Did the authors apply any segmentation algorithm and if not, why?

We entirely agree that the colour coding is confusing with respect to the convention for presenting over-expression in red and under expression in green. Unfortunately the colour coding in aCGH studies has been established as the opposite of that used in expression analysis, with red representing copy number loss, green representing copy number gain and yellow representing amplification. Please see Joe Gray’s papers on aCGH (Cancer Cell 2006 Chin et al. and Neve et al.) for further examples. We did not apply any segmentation algorithm because for tiling BAC arrays the response of each clone and its immediate neighbours is a highly reliable indicator of copy number and therefore visual analysis is not only sufficient but can be more accurate than segmentation analysis.

7. Concluding comments

The investigation performed by Cook et al is very thorough. They not only use a high density array platform but also take the time to investigate the organization of rearrangements by FISH or by flow sorted chromosomes, and by analysing primary tumours using TMA. The weakness of the manuscript is when the authors try to assign candidate genes for the rearrangements. Here the authors uses a mixture, and in many ways not well chosen, methods. The manuscript is also very “candidate gene oriented”. The authors should maybe step back for a moment and ask if there might be other possible explanations for their findings than selection for specific genes. In the introduction three tumour suppressor genes, or candidate regions, are mentioned. After the analysis of Cook et al this number has risen to five. Let’s say an additional cohort of tumours is investigated and further candidate regions are identified, would also these be interpreted as the location of tumour suppressor genes?

As described in response to specific comments above we have now included throughout the manuscript alternative explanations for the observed patterns of genomic change – namely the possible presence of breakage-prone fragile sites.

We have removed the reference to the five candidate regions present in DU4475 as only 3 of these are recurrent either within this dataset or in the context of the existing literature.

The final comment is again a very interesting point for discussion. Following the sequencing efforts of Vogelstein and others and as discussed by Beerenwinkel et al (PLoS computational Biology 2007) it is now being suggested that perhaps cancers can arise as the result of multiple mutations (they suggest 20 per tumour) each with potentially a small effect rather than as a consequence of a few high-impact changes. It certainly seems to be emerging that regardless of how many mutations are necessary to cause a tumour there will be multiple changes which have a modifying effect, and are therefore under selection – perhaps in these cases the classical terms of tumour suppressor gene and oncogene are misleading and describing them as cancer genes, as is done by Vogelstein gives a more accurate impression of their importance.
In summary, our aim for this manuscript was to provide a comprehensive genomic analysis of events on 8p, which would therefore provide a feasibly small number of candidate genes for functional studies if labs with the appropriate expertise wished to study their role in carcinogenesis. We carried out limited validation to clarify and prioritise candidate genes but accept that we have not definitively identified them as TSGs.

8. Minor Essential Revisions
Page 4. The authors mention only one tiling path study of breast cancer cell lines. The authors may also include Jönsson et al Genes Chromosomes and Cancer 46:543-558 (2007) that used the 32k set which basically is a tiling path set of BAC clones.
We appreciate our attention being drawn to this paper and have now included its finding of an 8p11-12 amplicon in JIMT-1 in our Introduction (p4).

Reviewer 2, Stephen P Ethier:

Minor Essential Revisions.

1. There is a wealth of data describing gene amplification at the 8p11-12 region in human cancers including breast, lung, colon, pancreatic and thyroid cancer. In previous study, the author’s group found that amplification of 8p11-12 occurs in 24% (8/33) of breast cancers (Ref 14). However, data obtained by other groups cited in this paper have found that in human breast cancer, focal amplification involving chromosome 8p11-12 occurs in approximately 10-15% in most published data. This should be mentioned in the paper as well.
We have made our statement about the 8p11-12 amplicon in the background section (p 4) more accurate by specifying that the 8p11-12 amplification has been found in 10-25% of breast cancers and included the appropriate references at this point (Gelsi-Boyer et al., Prentice et al., Ray et al.) as well as further on in the text

2. In the result section, the category subtitle “8p11-12” is not meaningful, I suggest the authors use: “8p11-12 amplicon, a novel amplicon in 8p21.3, 8p22 rearrangements, and loss of 8p22.3” as subtitles. The authors should delete the “DU4475” subtitle.
We agree that subtitles needed to be clearer and have changed them in line with this request. (p10)

3. As ARHGEF10A is a new candidate tumor suppressor gene that has a point mutation in the DU4475 line, screening and finding the same mutation in primary breast tumor will provide more convincing evidence for its tumor suppressor function.
This point was also raised by reviewer 1, comment 4 to which we gave the following response:
We entirely agree that finding multiple mutations in primary tumour material would be definitive in identifying ARHGEF10 as a tumour suppressor and that following our analysis it remains only a candidate gene. However, there are experimental obstacles to further mutation screening. Firstly, the quantity of primary tumour material we have available is limiting. Secondly, ARHGEF10 is a large gene requiring 28 PCRs and therefore a minimum of 56 sequencing reactions to sequence it in each sample. To do this on a number of primary samples is not trivial in terms of cost, time or the quantity of tumour DNA needed. Thirdly, identifying appropriate tumour samples to screen is not straightforward. In the absence of copy number data we cannot identify samples that have heterozygous deletions and are therefore most likely to contain point mutations. The low cellularity of breast tumours and the likelihood of heterozygosity at the locus mean that even at the site of a point mutation the normal sequence will be dominant on a sequencing trace and therefore mutations are hard to detect. The large-scale sequencing effort detailed in Wood et al. 2007 and Sjoblom et al. 2006 identified two heterozygous missense mutations in ARHGEF10, strengthening its status as a candidate cancer gene. We have included this point in our discussion.

Reviewer 3: Michael Krainer (corrections highlighted in grey on revised text)

1. As only minor essential revision I would like to have discussed the genomic results in relation to the receptors for TRAIL. The genes encoding these receptors (DR4, DR5 and the decoy receptors) are located in the area studied (8p21-22) and are discussed as potential tumor suppressor genes (intriguing by their function) by several groups. We have now included in the results (p 15) the statement that DU4475 has ‘an approximately 1 Mb deletion between 21.9 Mb and 23.0 Mb in 8p21, containing candidate cancer genes RHOBTB2 (22.9 Mb) and DR5 (Death receptor 5) (23.0 Mb) [40] but not extending as far as DR4’ and in the discussion (p20) ‘Potential targets [of DU4475 deletions] in 8p23.3 and 8p22 have been discussed above while 8p21, although only affected by a single small deletion in this study, is the location of RHOBTB2, a candidate breast cancer gene based on the discovery of two somatic mutations [40], and the TRAIL receptors DR4 and DR5, which have been suggested as candidate cancer genes owing to their pro-apoptotic function [60].’