Author's response to reviews

Title: Matrix-Comparative Genomic Hybridization from Multicenter Formalin-Fixed Paraffin-Embedded Colorectal Cancer Tissue Blocks

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Author's response to reviews: see over
Dear Ladies and Gentlemen!

Thank you for your letter concerning our abovementioned manuscript. All comments of the reviewer are addressed in this letter and if necessary have been implemented in the revised version of the manuscript (highlighted in red). We would like to thank the reviewer for these helpful suggestions, which have significantly helped to improve the manuscript.

In detail we have revised the manuscript as follows:

Major points:

Reviewer:
Since the study tests the suitability of FFPE tissues obtained from multiple institutions for analyzing the DNA copy number changes, it is relevant to indicate the year from which these FFPE tissues were collected. It is important to show what factors (for example, age of the block) probably affects the DNA and array quality.

Reply:
In table 1, we added one column with the date of sample preparation and one column with the DNA quality. Moreover, we show the cases in which the DNA quality was too bad. The age of the samples does not correlate with the tumor and array quality. This is also implemented in the manuscript.

Reviewer:
The authors state that best arrays were obtained using DNA that was moderately degraded (Figure 1; lane 3 and 7). It is puzzling to note that higher molecular weight DNA shown in lane 10 and 11 did not yield the best arrays. The authors should define the criteria to determine best quality arrays that are suitable for the analysis like what percentage of spots were readable and/or the background levels etc.

Reply:
The DNA in lane 10 and 11 is DNA from cell lines used as positive controls. It is not surprising that this DNA is not degraded like the others. These controls were shown in this figure to illustrate the grade of degradation of the tumor DNA. Array hybridizations with the test DNA were not performed.

In order to determine the quality of arrays that are suitable for the analysis, we used the specialized algorithm previously developed and validated by our group (for a more detailed explanation, see Nessling et al, Cancer Res 2005, 65: 439-447). In this algorithm, the quality of the Gauss fit was evaluated by Pearson’s R. The array experiments were declined if the R value of the fit did not reach 0.96. For clarification, we have added this information in the Material and Methods section (“Data Acquisition and Evaluation”) and in the Results section (“Quality of DNA…”).

Reviewer:
Statistical tests are inadequate; authors should show that the number of samples used for different tumor stages are statistically significant.
The reviewer is correct in remarking that the number of samples from each tumor stage is too low to allow definitive conclusions about statistically significant differences between the tumor stages. However, the main purpose of this study was to show the feasibility of performing matrix-CGH with DNA derived from FFPE samples. In addition, the results presented here show some interesting trends in the distribution of chromosomal aberrations, as discussed in the manuscript, and represent the basis for more detailed analyses using larger series of archived tissue samples.

It will be relevant to show an unsupervised cluster analysis of the array-CGH data. For example, based on the aCGH data obtained by the authors, it is possible that the samples 18 and 27 may be reclassified as UICC IV group.

Although cluster analyses of matrix-CGH data have been performed in a few studies, there is no consensus over the relevance of the clustering results and over the applicability of clustering algorithms to matrix-CGH data. I.e., it is unclear in which form (numeric values) aberration data should be entered into the clustering algorithms, how to weight high- and low-copy amplifications and deletions, respectively, which distance metric to use etc. The set of samples used in the present study is too limited in size to allow a systematic evaluation of these problems, but clustering of matrix-CGH data will be a very interesting research subject for more comprehensive data sets obtained in future studies.

FISH experiments using the BAC clones representing the proximal region of 18 Mb region will narrow down the disease genes.

At the time of these analyses, clone RP11-408E5 was the most proximal clone of chromosome 13q available from public resources. Even now there are only 5 new BAC clones described to be positioned proximal of this position (18.5 MB). Unfortunately, these only cover an additional 0.5 MB of the chromosome, so that additional FISH or CGH analyses could not be expected to contribute much more information.

Reviewer: Though the authors conclude that amplification of 13q region is associated with aggressive phenotype in colorectal carcinoma, no attempts were made to correlate/compare the aCGH data to the gene expression studies that are available for colorectal cancer to select candidate oncogene(s).

As mentioned above, the number of samples analyzed in this proof-of-principle study was too low to allow for meaningful statistical evaluation such as correlation with microarray data sets. In addition, a multitude of published microarray studies on colorectal cancer have produced vastly differing, and sometimes contradictory, results, so that it is unclear which array system and which data set(s) would be the correct one to compare our matrix-CGH data with.
to. In order to perform such an analysis, it would be necessary to simultaneously analyze a larger number of samples in genome and transcriptome microarray experiments. However, as mentioned in the manuscript, several of the genes in the candidate 13q region have been described as having important roles in several different tumors.

Minor points:
Reviewer:
Speculations as why 7 FFPE tissues samples failed to yield DNA will be appropriate for this study.

Reply:
The quality of DNA extracted from formalin fixed tissues could be influenced by several factors, mainly differences in fixation procedures. In the discussion of the manuscript we added a section addressing this point.

Reviewer:
Authors may consider using an alternative procedure like commercially available kits for extraction of DNA from the 7 FFPE tissues blocks from which no DNA was obtained.

Reply:
For DNA extraction we used a standard procedure with phenol-chloroform and proteinase k. Several methods were evaluated by us as well as collaborating groups, and this procedure proved repeatedly to be the best. The fact that the DNA extraction is not succesful in all cases is not surprising, as the integrity of nucleic acids in FFPE samples is well known to vary widely (depending for example on the formalin fixation procedure).

On an additional note, we would like to provide details concerning the statement of the ethical review board: The retrospective use of the tissue blocks for translational research was approved by the ethics committee of the "Ärztekammer Niedersachsen" (Berliner Allee 20, 30175 Hannover, Germany, reference number Grae/128/2002).

We hope that our revised version of the manuscript has addressed all concerns raised by the reviewer.

Sincerely yours

Thomas Gress