Author's response to reviews

Title: p53 expression is significantly correlated with high risk of malignancy and epithelioid differentiation in gastrointestinal stromal tumors. An immunohistochemical study with 104 GISTs.

Authors:

Ursula Pauser (upauuser@gmx.de)
Nina Schmedt auf der Günne (ninaschmedtadg@yahoo.de)
Günter Klöppel (gkloeppel@path.uni-kiel.de)
Hartmut Merz (merz@patho.uni-luebeck.de)
Alfred C Feller (feller@patho.uni-luebeck.de)

Version: 3 Date: 18 May 2008

Author's response to reviews: see over
Authors’ response to reviews

General Response:
We revised and rephrased the submitted article according to the Editor’s advice and the Reviewers’ suggestions. Language was corrected by the help of Dr. Helmut Zacharias. The requested ethical approval had been given by written consent of every patient, on which our retrospective study was based. Our departments do not maintain ethical review boards.

Former Title:
p53 expression is significantly correlated with high risk of malignancy and epithelioid differentiation in gastrointestinal stromal tumors. An immunohistochemical study with 104 GISTs

New Title:
P53 expression is significantly correlated with high risk of malignancy and epithelioid differentiation in GISTs. An immunohistochemical study of 104 cases

Authors:
Ursula Pauser, Nina Schmedt auf der Günne, Günter Klöppel, Hartmut Merz, Alfred C. Feller

Version: 2 Date: 15 April 2008
Reviewer: Luigi Insabato
Reviewer’s report:
Although authors showed some interesting data, there is also some dictation and grammatical error in the text.

Major points
1. Pag 5 immunohistochemistry parag. The authors sentenced :" Therefore, all cases in which the tumor cells showed a intensive positive nuclear reactivity for p53 were considered positive (cut off 0%)." Beside some grammatical error, I understand that only one positive nucleus should be considered pathological, and this is correct, however to be reasonable one should consider that tumours showing at least 5% of positive nuclei were considered for statistical analysis, for example. This should be written in the text.
Normal stromal tissue does not proliferate and remains unstained by P53 antibody (negative control). Therefore all GISTs, in which cells showed an intensive nuclear reactivity for P53, were considered positive.

2. Pag 5 to clarify the number of patients with follow-up. Do the authors get FU in 47 patients? It would be better to clarify which patients died for disease
Follow-up information was available on 49 patients. Ten patients died of the disease, all of them had metastases.

3. Pag 7 line 24 and line 25 “(3/16) of the GISTs with metastases (2 duodenal GISTs, 1 gastric GIST), but in 62% (8/13) of GISTs without metastases from various tumor sites” The number in bracket regarding GIST with and without metastasis is fairly confusing. The numbers should be the same reported in the material and methods and results paragraphs.
The main sentence reads now: Metastases had developed only in GISTs with intermediate (3/3) or high (17/27) risk of malignancy (p=0.001).
4. Photo 1 should be of better quality selecting a field with more nuclei positive. This criticism refers probably to Figure 1A. We confirm that it shows a representative P53 staining pattern. Therefore, we do not exchange the photo.

Minor points
Pag 2 last line epithlioid =epithelioid“ – Yes.
Pag 4 line 16 habe=have – Yes.
Pag 4 material and methods paragraph, line 4 “it is a retrospective study” to delete. The statement is essential for ethical approval. Rephased:
Every patient had given written consent before surgery that disease data shall be open for scientific evaluation. Therefore, our retrospective study was carried out in compliance with the Helsinki Declaration.
Pag 5 immuno parag, line 11 a=an – Yes.
Pag 5 same parag, line 12 intensive=intensive – Yes.
Pag 5 To specify the cut off of P53 bcl2 and cyclin D1 – Done.
Pag 6 results parag, line 12 kiS5=ki67 – Done.
Tab 1 the authors should add CD 34 antibody – Done.
Tab 1 KiS5=Ki67 – Done.

Version: 2 Date: 21 April 2008
Reviewer: Muna Sabah
Reviewer’s report:
The study is not novel and numerous studies have investigated the role of p53 immunostaining in the prognostigation of GISTs. The findings of this study are in concordance with those previously reported.

Major Compulsory Revisions
1- There is a new classification of GISTs by Miettinen which takes into consideration, tumour size, site and mitotic count. It classifies GISTs into 6 groups (1, 2, 3a, 3b, 4, 5, 6a and 6b). This classification is a combination between Fletcher classification and the old Miettinen classification. Therefore the old groups (probably benign, uncertain malignant potential and probably malignant) are not used anymore. By using an outdated classification, the authors may have drawn inappropriate conclusions. The authors should revise their findings taking into consideration the new classification.
This valuable piece of advice will be followed in future work. At the time that we designed the project, these standard classifications were used separately. Table 2 shows high malignancy risk with 55% and 54% P53 label in GISTs according to Fletcher’s and Miettinen’s criteria, respectively. The almost identical percentages do not foster the idea that the new, combined criteria would yield a greatly different result.

2- The authors have stated that that the epithelioid subtype of GISTs is known to be associated with an aggressive behaviour. This cellular pattern is present in one third of gastric tumours, but is usually associated with more aggressive behaviour when found in the small intestine. It would be useful to state how many of the GISTs with positive p53 staining and epithelioid morphology were gastric in origin and how many were non gastric.
With regard to epithelioid differentiation, 22 GISTs were P53 positive, of which 12 were localized in the stomach and 10 in the intestine.

3- The authors use the percentage 7% as a cut off point for Ki67 immunostaining, but have 0% as a cut off point for all other markers. They have reported that any positive staining is regarded
as positive. This is in contrast with other major p53 studies which use a minimum of 5-10% as a cut off point. We knew that other authors have used varying cut off levels for P53 from 1% to 50%. However, normal stromal tissue does not proliferate and remains unstained by P53 antibody (negative control). Therefore GISTs were considered positive when nuclei showed intensive p53 reactivity.

4- The poor punctuation and grammatical mistakes, made some paragraphs ambiguous. We complied with this advice.

Minor Compulsory Revisions
1- The authors have stated that 50% of GISTs are malignant, but fail to cite a reference for this percentage. This is a high percentage and a reference should be included. – Yes:
2- The authors have classified GISTs histologically into 2 groups only (epithelioid and spindle). Were there any other subtypes (mixed pattern which is seen in 20% of cases and pleomorphic 5%)?. Perhaps the cases in this study with severe cytological atypia should be classified as pleomorphic.
The tumor sections were classified in two groups according to the dominant pattern.
3- The authors have stated that “GISTs with metastasis expressed p53 more often than GISTs without metastasis”. Has this been seen in primary resection specimens or in the recurrence / metastatic sections? Was there a difference between primary tumours and their recurrences?
Immuoassays were performed with the primary tumors. Information on recurrence / metastasis came from the files of our departments.
4- The authors have discussed their findings on CD34 in the “discussion paragraph”, but there is no mentioning of CD34 in the “Material and Method” section nor in the “Result” section. – Done.
5- The incubation period of the antibodies used should be added to Table 1.
Added to Methods: Incubation with the primary antibodies at room temperature for 30 min.
6- Were appropriate positive and negative controls used in this study?
Added to Methods: Control tissues were added to every staining patch. The immunostaining of P53 was controlled particularly upon decorated nuclei in the mucous membrane, whereas normal stromal tissue, which does not proliferate, remained unstained (negative control).
7- c-kit protein expression should be referred to as KIT ( page 3 line 4 and page 4 line20). – Yes. Also done with the other antigens. Thanks for this valuable advice.
8- KIS5 should be replaced by KI67 (page 6 line 25 and table 1). – Yes.
9- There are some typing errors (e.g., page 4 line 16 should read as have rather than habe). – Yes; corrected.
10- Reference 12 is inappropriate for this study (Biankin) as it is about p21 which has not been investigated in this study. – Yes; deleted.
message of the paper. The English language in general is clear and correct although it needs some minor amendment.

Major Compulsory Revisions:
1) What were the actual criteria used for including a tumour into this study? From the Material and methods chapter it appears that a c-kit positive staining was obligatory. For a significant part of the time period 1973-2001 the diagnosis of GIST was not possible and therefore it needs to be clarified how the tumours were retrieved and how the GIST diagnosis was made. Our retrospective study used many primary gastrointestinal mesenchymal tumors from the pathology archives at Brussels, Varese and Kiel. Indeed, we identified the 104 GISTs by means of positive KIT staining those tissues.

2) Since the study attempts to evaluate staining results with regard to aggressive tumour behavior and patients survival it would be of interest to present Kaplan-Meier survival curves in order to demonstrate the separation of p53 positive and p53 negative cases. A Kaplan-Meier plot does not enhance insight, because only limited data on survival and presence of metastases were available.

Methods rephrased: Follow-up information was available on 49 patients. During a period of up to 284 months (mean 75), 33 patients survived disease free. Further 6 patients were still alive at the end of the follow-up, but sustained metastases. – Ten patients died of the disease, all of them had metastases. (Follow-up was not available for 5 patients who had metastases at the time of primary diagnosis.)

Results rephrased: Primary resections from GISTs with metastases showed P53 label more often than those without (65% vs. 39%). Survival analysis showed P53 positive tumors in 70% (7/10) of the patients who died, but only in 18% (7/39) survivals.

Minor Essential Revisions:
1) How were the tumours selected for the study? Were they all tumours of certain type in a specific pathology department? It would be interesting to have this clarified for epidemiological interests in an assessment of the data. 

Methods rephrased: We detected 104 KIT-positive tumors, 10 of which came from Brussels, 46 from Varese, and 48 from Kiel.

2) References are needed for a statement like this one: “Because 50% of GISTs become malignant…….” This is actually a debatable statement and should perhaps be rephrased accordingly. – Yes: Reichardt P, Hohenberger P: Gastrointestinale Stromatumoren (GIST). Bremen: Uni-MED 2006.

3) Much of what is being written within the background (introduction) chapter should preferably be presented within the Material and Methods chapter. There is a rather detailed description of what actually was done in this study presented within this chapter of the manuscript. This should preferably be presented in the Materials and Methods chapter and possibly shortened. – Done.

4) Each tumour apparently was evaluated by two pathologists with regard to assessing certain pathological parameters. How was that actually performed? Did they evaluate the specimens together with a joint decision or separately? If the latter was the case how was the final decision made? 

Methods rephrased: Without knowledge of the clinical data, UP and NSG evaluated independently all tumor specimens by light microscopy. Most of the judgments agreed; doubtful cases were scrutinized once more or discarded.

5) It needs to be added to the Material and Methods chapter that mitoses were evaluated as other pathology parameters.

The mitotic rate was mentioned in Methods and detailed in the beginning of Results.

6) CD34 staining is being reported in the results chapter although not mentioned in the Material and Methods chapter. – Done: Methods rephrased.

7) The conclusions in the main text should not imply an association between
positive p53 staining and intestinal location since this did not prove significant in the paper. – Done.

Discretionary Revisions:
1) Frequently sentences are being initiated by a numerical number. In general I would attempt to rephrase these sentences and avoid that. – Done.
2) I would recommend rephrasing the first sentence of the conclusions both in the abstract and the main text. – Done.

Version: 2 Date: 26 April 2008
Reviewer: Sonja E. E Steigen
Reviewer's report:

Major compulsory revisions: There seems to be no need for major compulsory revisions.

Minor essential revisions:
Background:
- first paragraph - the authors state that 50% of the GISTs become malignant. GISTs are never really benign lesions, and a clarification about malignancy must be stated. Patients who relapse/get metastatic disease? Also references to this.
Yes: Metastases, relapse and dead are understood as criteria of malignancy [Reichardt P, Hohenberger P: Gastrointestinal Stiomatumoren (GIST). Bremen: Uni-MED 2006].
- first paragraph - Histological differentiation and tumor localisation correlate with prognosis. Why not refer to Hornick and Fletcher 2007 and USE this for further grouping of the GISTs according to malignancy? – Done.
- first paragraph - little information about development of the malignancy potential.... You should maby refer to studies on p16 and GIST even though they are controversial. p16 is regarded as cell cycle regulator. Schneider-Stock et al 2005, Steigen et al 2008. Further mitotic regulators (e.g. P21) could be including with the reviewer’s argument, but we had decided for KIT, CD34, Ki67, P53, BCL-2 and Cyclin D1.

Material and methods:
Immunohistochemistry
What antibodies did you stain with? Stained the slides with KIT? You stained with CD34 because you say so later in the paper. Suddenly also Ki67 is mentioned, but has not been mentioned earlier. Should probably have been mentioned earlier as this is improtant for diagnosis. Did you only use KIT+ tumors? Clarify! – Done.

Results:
- first paragraph - the results of KiS5 are revealed. No previous information about KiS5. An antibody against Ki67 epitope? Did you stain with Ki67 or KiS5? Must be clarified. – Done (Table 1):

Discussion:
- first paragraph - Too bad no mutation analyses have been done – mutations have been refered to several times how important they are.
Rudolph P: c-kit mutations, cell proliferation and prognosis in gastrointestinal stromal tumors (GISTs). Path Res Pract 2002: 198: 138-139.) Exons 9, 13 and 17 of c-kit and PDGFRα gene were not investigated. Others published conclusive results in meantime.

-second paragraph - expression WERE not...., all three markers WERE..... – Done.

Discretionary Revisions:
Be updated on other studies. – Yes.