Author's response to reviews

Title: QPRT: a potential marker for follicular thyroid carcinoma including minimal invasive variant. A gene expression, RNA and immunohistochemical study.

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Author's response to reviews: see over
Dear Madam,

In the accompanying files please find our revised manuscript

“QPRT: a potential marker for follicular thyroid carcinoma including minimal invasive variant. A gene expression, RNA and immunohistochemical study.”

by

Nora Hinsch, Matthias Frank, Claudia Döring, Christian Vorländer, Martin-Leo Hansmann.

According to your editorial points we

1. added the sentence “All specimen were originally submitted for diagnostic purposes and studied in accordance with national ethical principles and in compliance with the Helsinki declaration. Informed consent for the use of fresh frozen material in gene expression analysis was obtained from the patients. The study was approved by the ethics committee of the university hospital Frankfurt/Main.” at the end of the first subchapter of material and methods.

2. The abstract has been rewritten and now reads:

“Background: The differential diagnosis between follicular thyroid adenoma and minimal invasive follicular thyroid carcinoma is often difficult for several reasons. One major aspect is the lack of typical cytological criteria in well differentiated specimens. New marker molecules, shown by poly- or monoclonal antibodies proved helpful.

Methods: We performed global gene expression analysis of 12 follicular thyroid tumours (4 follicular adenomas, 4 minimal invasive follicular carcinomas and 4 widely invasive follicular carcinomas), followed by immunohistochemical staining of 149 cases. The specificity of the antibody was validated by western blot analysis.

Results: In gene expression analysis QPRT was detected as differently expressed between follicular thyroid adenoma and follicular thyroid carcinoma. QPRT protein could be detected by immunohistochemistry in 65% of follicular thyroid carcinomas including minimal invasive variant and only 22% of follicular adenomas.

Conclusions: Consequently, QPRT is a potential new marker for the immunohistochemical screening of follicular thyroid nodules. “

3. The raw data is currently in the submission process at Gene Expression Omnibus.
4. A “Competing interests” section has been added: “The authors declare they have no competing interests.”

5. The manuscript has been copyedited to improve the style of written English and formatted so that the submission conforms the journal style.

We hope that the manuscript is now acceptable for publication in BMC Cancer.

Sincerely,

Nora Hinsch
Responses to the reviewers.

Juan Pablo Rodrigo

The reviewer criticises that the clinical relevance of our findings is low. The histological differentiation of follicular thyroid carcinoma and adenoma is, however, still difficult and of high clinical relevance. The postoperative treatment of patients with a follicular adenoma and a follicular carcinoma differs widely, and patients, in whom the diagnosis of a follicular carcinoma is missed, do not benefit from oncologic treatment and have a higher risk of recurrences and distant metastases. As the reviewer mentions himself, an important further application for our marker could be the immunocytochemical staining of fine needle aspirates. Due to the low incidence of follicular thyroid carcinomas we are currently sampling material from fine needle aspirates for further experiments.

The reviewer criticises the low sensitivity and specificity of our new marker, compared to the markers described in literature. The responses to point 5 and 6 include a reply to this concern raise by the reviewer.

Major points

1. The reviewer suggests to clearly state which positive and negative controls were used in immunohistochemical analysis. Therefore we added the sentence „tonsils with follicular hyperplasia where used as positive controls. For negative controls, no antibody was added“ at page 5, line 5 in the manuscript.

2. The reviewer recommends to state the staining pattern. Therefore we added the sentence „The staining pattern was heterogen. The percentage of positive cells was estimated in relation to negative tumour cells.“ at page 5, line 12 in the manuscript.

3. The reviewer emphasises that the data presented in table 2 and figure 2A is the same. We eliminated figure 2A and changed figure 2 (see next section).

4. The reviewer recommends so demonstrate if there was an agreement between western blot, qRT-PCR and immunohistochemistry. Figure 2 now shows the immunohistochemical staining of three adenomas and carcinomas. Figure 3 highlights the results of western blot analysis and qRT-PCR of the same cases. “Legend to figures” now reads:
**Figure 2:** Representative picture of immunohistochemical staining of FTC (A-C) and FTA (D-F). Carcinoma tissue displays a cytoplasmatic staining of the tumour cells, while adenoma tissue remains unstained.

**Figure 3:** qRT-PCR (A) and western blot analysis (B) of the 3 FTA and FTC presented in figure 2. In qRT-PCR, carcinomas reveal a relative quantity of QPRT-RNA expression between 3.65 and 25.18. The relative quantity of QPRT-RNA expression in adenomas is between 0.13 and 1.00. In western blot analysis, FTC reveal a strong band at 34kD, while follicular adenomas lack any band. Blotting with actin was performed as loading control. Ad: Adenoma; Ca: Carcinoma.

The 11th line in the “immunohistochemistry” paragraph in the results section was changed to: “Results are shown in table 2. Figure 2 shows an example of immunohistochemical staining.”

In addition, we changed the data presented at the section „qRT-PCR“. The data presented in the first version of the script was a comparison of QPRT-expression in FTC with surrounding normal thyroid tissue. Now we present a comparison between four FTC and four FTA. Therefore we changed the sentence in the 5th line of the material and methods section to “qRT-PCR: Fresh frozen material from 4 FTA and 4 FTC was used.”. The sentence in the 5th line of the second paragraph in the material and methods section: ”RNA extraction from formalin-fixed, paraffin embedded material was performed by using Ambion RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, An Applied Biosystems Business, Austin, USA), following the manufacturer’s instruction.” was deleted. The second line of the paragraph “TaqMan® Quantitative real-time PCR” in the methods and materials section was changed to “Beta-2-microglobulin (B2M) was used as endogenous control (4326319E, Applied Biosystems) for relative quantification.” The paragraph “qRT-PCR” in the results section was changed to “qRT-PCR of FTC compared to FTA showed a ΔΔCt of 10.44 in FTC compared to 0.42 in FTA, indicating a more than twenty times higher amount of QPRT-RNA in carcinoma tissue (Figure 3A).”

5. The reviewer suggests to clarify that our marker does not identify malignancy but that it could help in the diagnosis of FTC. The sentence in line 5 of the discussion was changed to “In further immunohistochemical validation QPRT turned out to be helpful in discriminating between FTA and FTC.” Furthermore, we added the
sentence “The potential value of this marker lies in the screening of thyroid nodules, with nodules staining positively being processed intensively.” in line 11-13 of the discussion.

We did not add further comments concerning other markers because this is subject of the second section of the discussion.

6. The reviewer criticises, that important references about molecular markers used in the diagnosis of follicular neoplasms are lacking. The references mentioned, however, by the reviewer use a different methodological approach than applied by us. Benign and malignant thyroid nodules were compared without differentiating between follicular and papillary carcinoma. In contrast, we explicitly investigated only follicular thyroid carcinomas and adenomas, without including oncocytic adenomas. The total number of follicular carcinomas included in the study from Bartolazzi et al. is only 15, without differentiating between minimal invasive and widely invasive. In addition the number of follicular carcinomas included in the studies investigated by Sanabria et al. is not mentioned. Therefore these thorough examinations of galectin-3 in fine needle aspirates are not adaptable for our discussion.

7. The reviewer demands, that the limitations of the study should be clearly specified in the conclusions. Therefore, the conclusion was changed to „In conclusion, gene expression analysis revealed a new immunohistochemical marker, which may be helpful in differentiating FTC from FTA. QPRT is a potential useful marker for immunohistochemical screening analysis of solitary thyroid nodules. In case of positivity the lesion should be processed extensively. This procedure probably reduces the number of misdiagnosed or overlooked minimal invasive carcinomas. The sensitivity of the new monoclonal antibody is, however, limited. For this reason, QPRT as other new markers has the highest diagnostic relevance if it is applied not solitary but in combination. Additional prospective studies of fine needle aspirates should be included. “

Minor points
The reviewer proposes to upload table 1 and 3 as pdf-files. Unfortunately, tables 1 and 3 are in landscape format and therefore not includable in the body of the article. Hence they are presented as additional files.

Discretionary Revisions
1. The reviewer demands an indication of the primers used. We used TaqMan® gene expression assays from applied biosystems (the part number is given in the manuscript). The primers used in the assay are not indicated by applied biosystems.

2. The reviewer proposes to include the positive and negative predictive values. Accordingly, we added the sentence „The positive predictive value is 0.73, the negative predictive value is 0.71“ to the second paragraph of the results section.

3. The reviewer criticises, that we indicate, that the results of gene expression analysis are more platform dependent than tissue-dependent. The results are, however, also platform-dependent as probe sets are not identical between different providers of these assays. Therefore expression patterns of individual genes might vary. The overall expression patterns results are, however, comparable between different platforms.
Kenneth Devaney

1. The reviewer suggests to edit the grammar of the manuscript. The manuscript has been copyedited to improve the style of written English and several changes have been made.

2. The reviewer emphasizes that our marker will probably be most useful as one component of a panel. Accordingly we changed the sixth sentence of the conclusions section to “For this reason, OPRT as other new markers has the highest diagnostic relevance if it is applied not solitary but in combination.”