Author's response to reviews

Title: Decrease in thyroid adenoma associated (THADA) expression is a marker of dedifferentiation of thyroid tissue

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Author's response to reviews: see over
Enclosed please find the revised version of our manuscript entitled "Decrease in thyroid adenoma associated (THADA) expression is a marker of dedifferentiation of thyroid tissue". We have addressed all criticisms and suggestions made by the reviewers (bold letters).

Reviewer: Giuseppe Damante

Major criticism.

Authors claim that THADA is a marker of dedifferentiation of thyroid tissue. They support this statement with two observations:

- THADA is reduced in anaplastic carcinomas compared to other (more differentiated) thyroid neoplasms.

- By comparing the present results with those of HMGA2 expressions (previously published), they found a significant negative correlation. Both these issues are quite weak to demonstrate that THADA expression is a marker of dedifferentiation in thyroid tissues. Authors should correlate THADA expression of papillary and follicular carcinomas (in which the degree of differentiation is quite heterogeneous) with a very much recognized marker of thyroid differentiation (i.e. NIS or TPO gene expression).

To address that helpful suggestion, now forty-one thyroid FFPE samples were checked for the expression of NIS, including seven normal tissue samples, six nodular goiters, five adenomas, and 23 carcinomas (15 PTCs, four FTCs, and all four ATCs). A Spearman's rank correlation was then used to determine the relationship between the expression data of THADA and NIS and revealed a significant correlation.
Minor criticisms.

1. Page 7, line 7. “18S rev_1” should be “reverse”.

‘18S rev_1’ is the name of the primer as found in Antonov et al. (2005) [24 in the manuscript]. It would be unclear which primer we used if we would refer to it simply as ‘reverse’ since Antonov et al. used three different 18S reverse primers.

2. Page 8, line 8 from the bottom. Source of snap-frozen tissues must be better specified; “taken for diagnostic purposes” is not sufficient.

During pathological examination, a sample of the tissue was snap-frozen. The procedure was approved by the local ethics committee (Ethikkommission bei der Ärztekammer Bremen). The corresponding passage has been stated more precisely.


The typing error has been corrected.

4. Page 11, paragraph “transcription factors binding to THADA”. Since the transcription factor HEX plays a role in thyroid-specific gene expression, information on the presence of putative HEX binding sites is required.

Aimed at putative HEX binding sites we have performed in silico analyses. HEX is not included in the SABiosciene DECODE Transcription Factor Search and thus we used the programming language Perl in an otherwise manual search. The DNA binding site of Proline-Rich Homeodomain protein (PRH/HEX) was analyzed in the promoter region of the THADA gene. The consensus sequence 5’ c/t a/t ATTAA a/g ‘ as determined by SELEX (systematic evolution of ligands by exponential enrichment) experiments and DNase I footprinting assays (Soufi and Jayaraman, *Biochem J* 2008, 412:399-413; Williams et al., *J Mol Biol* 2008, 383:10-23) was found two times in the THADA promoter region. Given the frequent random occurrence of the consensus sequence of HEX sites throughout the genome (each 8,192 bp) and under the assumption that the HEX transcription factor binds DNA as an oligomer using multiple binding sites it seems not likely that these are real binding sites. Other identified binding sites (5’_TAAT-3_, 5’_CAAG-3_ or 5’_ATTAA-3_ in EMSAs (electrophoretic mobility-shift assays), Soufi and Jayaraman, 2008; Williams et al., 2008)) are too short and therefore too frequent for an analysis. In a shortened form, this information has been added to the corresponding passage.
5. Page 12, line 11. Authors state “clear indication for a role of THADA in maintaining thyroid differentiation is presented”. These statement is not correct; authors show evidence THADA expression “is associated to thyroid differentiation” (provided that the major criticism is addressed).
To give a more appropriated statement the sentence has been rephrased as suggested.

Reviewer: Vasyl Vasko

1. Introduction.

a The hypothesis is not clearly stated:

i. the functional role of THADA in regulation of thyroid cell differentiation?
ii. the role of THADA in apoptosis?
iii. The role of THADA in normal thyroid development or tumorogenesis?
iv. Association of THADA expression and diabetes?

In the manuscript we stated that there was no information available about the function of THADA except for a purported interaction with death receptor DR5 which has been mentioned including a brief hypothesis about the possible role of THADA in apoptosis. To the best of our knowledge our results give the first evidence for a certain role of THADA in the thyroid other than the involvement in adenomatous growth (Rippe et al., 2003, [3 in the manuscript]). Because of this, we do not feel it would be appropriate to give a clear hypothesis in the introduction of the role of THADA in regulation of thyroid cell differentiation, nor in normal thyroid development or tumorigenesis.

As mentioned in the manuscript, THADA has been linked to type 2 diabetes. There are several publications concerning this subject [5-20 in the manuscript], which we have now briefly summarized.

2. Methods

a. Please provide clinico-pathological data of patients with thyroid cancer. The information on functional activity of thyroid adenomas will be useful.

Unfortunately, no information about the functional activity of the thyroid adenomas was available. However, in the manuscript we have now included a table with the clinico-pathological data of the patients with thyroid cancer.
b. Thyroid cancer cell lines are listed in method section. However there are no data on THADA expression in thyroid cancer cell lines in results section of manuscript.

The thyroid cancer cell lines were used for the assay validation and were mentioned in the according passage. Since we feel this paragraph might be misleading it has been deleted. Please, see also 3.a.

3. Results

a. The section “Assay validation” is too detailed.

The assay validation has been shortened and included in the corresponding passage of the materials and methods section.

b. THADA expression in normal tissues. There is discrepancy between the data presented in Fig 1 and statement on sensitivity/specificity.

To address this important point raised by the reviewer, our statistician rechecked the numbers and found them to be correct. As pointed out in the manuscript, sensitivity, specificity and decision limits were calculated from non-parametric density estimations. Therefore, sensitivity and specificity may differ from raw empirical values (which might be read from the graphs) and decision limits need not coincide with measured values. The latter explanation has been inserted into the text.

c. THADA expression in thyroid tumors

i. Provide p value for the difference between the level of THADA expression in normal thyroid and benign lesions (goiter, adenomas).

Please, see comment 3. c. ii.

ii. Provide p value for the difference between the level of THADA expression in normal thyroid and differentiated papillary thyroid cancer.

The values for the difference between the level of THADA expression in normal thyroid and benign lesions and in normal thyroid and differentiated papillary thyroid cancer have been added to the manuscript.
iii. Provide data on expression of well established thyroid differentiation markers (NIS, TG, TPO and other) in relation to THADA expression.

Please, see reviewer 1 - major criticism.

iv. Which genetic abnormality were detected in examined anaplastic thyroid cancer

In our institute one of the anaplastic thyroid samples served as the source of a newly established cell line. Cytogenetical analysis revealed a highly complex karyotype with a range of 80 to 117 chromosomes (average: 100.8). Several marker chromosomes, telomeric associations, and double minutes were detected. The composite karyotype reads as follows: 80~108<4n+>,XXXX,-Xx2[3],1x2[6],+der(1)t(1;?)(p11;?)[3],+der(1;2(q10;p10))[2],+der(1;8)(p10;p10)[5],-2[3],+3[6],-4x3[4],+der(4;14)(p10;q10)x2[6],+5[3],+dic(6;?)[2],+7[6],+7[4],+add(7)(q11.2)[2],-8[5],+8[2],+der(8)8;?)(q10;?)[3],-9[5],+add(9)(p11)[3],+der(9)t(2;9)(q11;p13)[4],-10x2[7],i(10)(q10)[7],+11[3],+del(11)(p11)[4],-12[4],-13[5],-14x4[4],-4x3[3],+der(14)t(14;?)(p11;?)[3],+der(14)t(14;19)(p11;?)[3]+dic(14;14)(q10;q10)[4],-15x2[5],+del(15)(q24)x2[6],+16[3],-17[6],-1[5],-19x2[5],+add(19)x2[3],+add(19)[5],+20[5],-22x3[4],-22x4[3],+13-21mar[7].

This information (in an abbreviated form) has been added to the manuscript (section methods, subsection tissue specimen and RNA isolation). Unfortunately, for the other three samples no such information is available.

v. The results on HMGA2 expression were already reported. It is unclear how you performed correlation analysis using data from previous study.

Indeed, the same 41 samples were used in the present study as well as in a previous investigation (Belge et al., 2008, [32 in the manuscript]). RNA was isolated from adjacent cuts of the same FFPE block. Subsequent sections were analyzed by qRT-PCR, one for THADA, one for HMGA2 under otherwise identical conditions (i.e. using the same kits, protocols and lab equipment). A Spearman's rank correlation was then used to determine the relationship between the expression data of the two genes.

The corresponding paragraph has been stated more clearly.
d. Transcription factor binding.

i. Define in more detail the functional relationships between THADA and CREB

The SABiosciences DECODE Transcription Factor Search shows only possible binding sites, in this case for CREB in the THADA promoter, but does not give further information, nor is it available in the literature. The functional interaction between THADA and CREB would be of interest in future research.

We hope that we have sufficiently addressed all comments, suggestions and criticisms and that the paper is now suited for publication in **BMC Clinical Pathology**. Thank you very much in advance for your consideration.

With kind regards,

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