CLINICAL STUDY

Characterisation of *DEHAL1* expression in thyroid pathologies

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Abstract

Iodotyrosine dehalogenase 1 (DEHAL1) is a transmembrane protein involved in the recycling of iodide in the human thyroid. The aim of the present study was (I) to investigate whether DEHAL1 expression is different in differentially functioning thyroid pathologies and (II) to evaluate DEHAL1 as a possible marker of thyroid cell differentiation.

Design and methods: Real-time PCR for DEHAL1 and its isoform DEHAL1B was performed in a series of 105 thyroid specimens, including toxic thyroid nodules (TTN), Graves' disease (GD) thyroids, benign cold thyroid nodules (CTN), normal thyroid tissues and thyroid cancers (follicular thyroid carcinomas (FTC), papillary thyroid carcinomas (PTC), partially differentiated thyroid cancers (PDTC) and anaplastic thyroid carcinomas (ATC)). In addition, DEHAL1 protein expression was studied by immunohistochemistry in 163 benign and malignant thyroid pathologies and normal thyroids.

Results: (I) The highest *DEHAL1* mRNA levels were found in GD thyroids, while downregulation of *DEHAL1* and *DEHAL1B* mRNA occurred in PTC and ATC ($P < 0.001$ and < 0.05 respectively); (II) DEHAL1 protein was overexpressed in TTNs and GD thyroids with predominant apical staining in all samples; (III) a weaker and patchy staining pattern was found in CTNs and normal thyroids; (IV) in differentiated thyroid cancers (FTC and PTC), a diffuse cytoplasmic DEHAL1 expression was found; and (V) in PDTC and ATC, DEHAL1 expression was faint or absent.

Conclusion: Upregulation of DEHAL1 protein expression and sublocalisation of DEHAL1 at the apical cell pole in TTNs and GD thyroids is consistent with increased thyroid hormone turnover during thyrotoxicosis. Diffuse cytoplasmatic localisation or downregulation of DEHAL1 expression in thyroid cancers suggests alteration or loss of DEHAL1 function during thyroid cell dedifferentiation.

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Introduction

Iodotyrosine dehalogenase 1 (DEHAL1) has been identified as a thyroid protein with a putative molecular size of 33 kDa (1–4).

The DEHAL1 is involved in the NADPH-dependent deiodination of the free mono-iodotyrosines (MIT) and di-iodotyrosines (DIT), which are released along with the thyroid hormones T4 and T3 through thyroglobulin proteolysis. DEHAL1 action on DIT and MIT results in the liberation of iodide and tyrosine, which can be reused for thyroid hormone synthesis.

The gene coding DEHAL1 has recently been cloned and is localised on chromosome 6 (6q24-25) (5). Besides the thyroid, *DEHAL1* mRNA has been found in human kidney and liver tissue, albeit at lower expression levels. In addition, a variant gene product termed DEHAL1B has been described, which differs from

the DEHAL1 isoform by a 102 bp insertion and is assumed to be functionally inactive (4, 5).

Gnidehou *et al.* (4) recently described first structural and functional properties of DEHAL1. They proposed that it constitutes a transmembrane protein with a large extracellular N-terminus containing most of the nitroreductase domain, a single transmembrane segment and a short intracellular C-terminus. Furthermore, they suggested that DEHAL1-catalysed deiodination takes place at the apical membrane in close proximity to the thyroglobulin iodination site. *DEHAL1* mRNA expression is upregulated by cAMP, which implies that DEHAL1 along with the sodium-iodide symporter (NIS), thyroid peroxidase (TPO) and thyroglobulin (TG) may represent a marker of differentiated thyroid function.

Since intrathyroidal iodine metabolism is known to vary considerably between the euthyroid and

hyperthyroid state as well as in thyroid pathologies with different functional properties, e.g. toxic and cold thyroid nodules (6, 7), we asked to what extent this may be reflected by differences in *DEHAL1* expression. Furthermore, we asked whether *DEHAL1* expression was altered with ongoing thyroid dedifferentiation.

Materials and methods

Thyroid samples

A total of 16 benign cold thyroid nodules (CTN; 6 colloid nodules and 10 follicular adenomas), 10 toxic thyroid nodules (TTN; 7 colloid nodules and 3 follicular adenomas), 26 surrounding normal thyroid tissues (STs) of the same patients, 8 Graves' disease (GD) thyroids, 21 follicular thyroid carcinomas (FTC), 18 papillary thyroid carcinomas (PTC) and 6 anaplastic thyroid carcinomas (ATC) were studied for *DEHAL1* and *DEHAL1B* mRNA expression.

In addition, paraffin-embedded tissue sections of 163 thyroid specimens (partially including the samples analysed for mRNA expression) were used for immunohistochemical studies. These comprised 16 CTN and 11 TTNs with corresponding normal tissues, 5 GD, 8 FTC, 63 PTC (17 follicular variant PTC, 13 conventional PTC and 34 tall cell variant PTC), 22 partially differentiated thyroid cancers (PDTC), 6 ATC samples and 5 medullary thyroid cancers (MTC; negative controls).

Thyroid samples were obtained from the Department of Surgery, Martin-Luther-University of Halle, Germany, local Leipzig hospitals and the Department of Pathology and Neuropathology, University Hospital of Essen, Germany. Thyroid samples were selected by corresponding results for ultrasound, scintiscan and histology as described (8, 9). Patients with TTN or GD received antithyroid medication and had euthyroid function or subclinical hyperthyroidism at time of surgery. Patients with CTNs, FTC, PTC and ATC did not receive thyroid medication and were euthyroid at time of surgery. Informed consent was obtained from all patients. The local ethics committee approved the study.

RNA extraction and RT-PCR

Snap frozen tissue samples were pulverised and transferred into TRIzol reagent (Invitrogen) for RNA extraction. RNA cleanup was performed using the RNeasy Mini Kit 50 (Qiagen Sciences). cDNA synthesis was performed at 37 °C for 60 min with 1 µg RNA using random hexamer primers (Promega) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Consistent expression of housekeeping genes (β -actin (*ACTB*) and *GAPD*) was demonstrated in all samples by RT-PCR as described (8, 9).

Real-time PCR

Real-time PCR was performed using the LightCycler System and LightCycler-DNA Master SYBR Green I Kit (Roche) according to the manufacturers' instructions: annealing temperatures and $MgCl_2$ concentrations were optimised to create a one-peak-melting curve. The following intron-spanning sense and antisense primers were used:

DEHAL1 : forward : 5'-TTT CCT GAC TCC CAT CTT GG-3'

reverse : 5'-TGC CAT TCA TCA GCA TCT TC-3'

DEHAL1B : forward : 5'-TCA CCA TGC GGC ATC AGA CT-3

reverse : 5'-CCA AGA GGA GCA GGC GAG AGG-3'

After initial denaturation (30 s) at 95 °C, PCR was carried out for 40 cycles (95 °C for 0 s, 64 °C for 7 s and 72 °C for 27 s, 4 mM $MgCl_2$) for *DEHAL1* and for 40 cycles (95 °C for 0 s, 62 °C for 7 s and 62 °C for 27 s, 4 mM $MgCl_2$) for *DEHAL1B*. The LightCycler software calculated the threshold cycles. Each sample was measured in duplicates and real-time PCR was repeated at least once. The fold difference (*n*) in upregulation or downregulation of mRNA expression was calculated as follows:

$$n = \frac{2^{(\text{threshold cycle gene (NT)} - \text{threshold cycle gene diseased tissues (DT)})}}{2^{((\text{ACTB (NT)} - \text{of ACTB (DT)})}}$$

'Normal tissue' corresponds to the surrounding tissue of toxic thyroid nodules (for TTN and GD) and cold thyroid nodules (for CTN, FTC, PTC and ATC) respectively. The Mann-Whitney *U*-test within the SPSS software version 11.5 was used for statistical analysis.

Immunohistochemistry

Paraffin embedded sections (3 µm) were dewaxed and rehydrated. Subsequently, sections were pretreated in a microwave oven in 0.1 M citrate buffer (pH 6) for one cycle at 750 W for 3 min and for 4 cycles of 3 min at 350 W. The LSAB+ System (DAKO Cytomation; Hamburg, Germany) was used for immunodetection. Briefly, slides were incubated with 3% H_2O_2 for 30 min followed by three wash steps with PBS/1% BSA. Unspecific binding was blocked for 30 min, and slides were incubated with a polyclonal *DEHAL1*-specific antibody as previously described (4). Immunoreactivity was demonstrated using a biotinylated secondary anti-rabbit antibody, streptavidin peroxidase and diaminobenzidine. Sections were counterstained with hemalaun and mounted in Aquatex (Merck). Semiquantitative analysis of *DEHAL1* staining was done by three

Table 1 Median mRNA expression levels for *DEHAL1* and *DEHAL1B* in different thyroid pathologies.

Samples	DEHAL1	DEHAL1B
Graves' disease (GD, <i>n</i> =8)	2.08	0.95
Toxic thyroid nodule (TTN, <i>n</i> =10)	1.36	0.70
Surrounding tissue of TTN (stTTN, <i>n</i> =10)	0.99	0.86
Cold thyroid nodule (CTN, <i>n</i> =16)	1.71	2.99
Surrounding tissue of CTN (stCTN, <i>n</i> =16)	0.84	0.70
Follicular thyroid carcinoma (FTC, <i>n</i> =21)	0.83	1.04
Papillary thyroid carcinoma (PTC, <i>n</i> =18)	0.26*	0.06*
Anaplastic thyroid carcinoma (ATC, <i>n</i> =6)	0.05†	0.06†

Results show fold upregulation (median) of *DEHAL1* and *DEHAL1B* expression in thyroid pathologies compared to normal thyroid tissues (stTTN for TTN and GD; stCTN for CTN, FTC, PTC and ATC). *Significant differences in *DEHAL1* and *DEHAL1B* expression were found in PTCs ($P<0.003$ and $P<0.0001$ respectively) and †ATCs ($P<0.001$ and $P<0.05$ respectively; Mann–Whitney *U*-test) compared with all other thyroid specimen.

independent investigators (S K/S Y S/D F) by determining the percentage of positively stained thyrocytes/six randomly selected sections/slide (200× magnification) and quantified as follows: –, no staining; +, <30% staining; ++, 30–60% staining; +++, >60% staining.

Results

Analysis of *DEHAL1* and *DEHAL1B* mRNA expression in thyroid pathologies

DEHAL1 gene expression was detected in all thyroid tissue samples (Table 1; Fig. 1A). The highest *DEHAL1* mRNA levels were found in GD thyroids. Both TTN and CTN showed higher median *DEHAL1* mRNA levels in comparison with the surrounding normal tissues (ST), but this did not reach statistical significance (Fig. 1A). There was no difference in *DEHAL1* mRNA expression between the ST of CTN and TTN. Downregulation of *DEHAL1* mRNA was found in PTCs ($P<0.003$) and ATCs ($P<0.001$; Table 1; Fig. 1A).

We then asked whether these changes in *DEHAL1* mRNA expression could be due to altered splicing. In CTN and FTC, we found an upregulation of the *DEHAL* isoform 1B reaching significance ($P<0.001$) for CTNs (Table 1, Fig. 1B), while *DEHAL1B* was unchanged in the hyperfunctional tissues and their ST. In PTC and ATC, *DEHAL1B* was downregulated or absent ($P<0.0001$ and $P<0.05$; Table 1; Fig. 1B).

Analysis of *DEHAL1* protein expression in thyroid pathologies

Immunohistochemical analysis showed a very strong, uniform *DEHAL1* staining at the apical pole in all GD thyroids and TTN samples (Figs 2A and B and 4A). A much weaker cytoplasmic staining pattern, predominantly found in small follicles, was observed in CTN (Fig. 2D) and all STs (Figs 2C and E and 4A). There were no differences in *DEHAL1* staining intensity between the ST of TTN and CTN. A diffuse cytoplasmic staining pattern of variable intensity (weak to strong, Fig. 4A) was found in FTC (Fig. 2F) and PTC (Fig. 2G). No significant differences were detected for PTC subtypes (follicular, tall cell and conventional PTC, Fig. 4B). In partially differentiated and undifferentiated thyroid cancers, *DEHAL1* protein expression was weak (9/22 PDTC) or absent (13/22 PDTC; Fig. 3B, all ATC; Fig. 2H). No *DEHAL1* protein expression was detected in five medullary thyroid cancers, which were used as negative controls (Fig. 2I). Results of the semiquantitative analysis of *DEHAL1* protein expression (relative distribution of staining/thyroid specimen in different subgroups) are shown in Fig. 4A and B.

Discussion

In order to investigate a possible alteration in the expression of iodotyrosine dehalogenase 1 due to

Figure 1 Expression of *DEHAL1* and *DEHAL1B* mRNA in different thyroid pathologies. Box plots showing median and distribution (box area=50% of samples) of *DEHAL1* mRNA expression (A) and *DEHAL1B* mRNA expression (B) in different thyroid pathologies and normal thyroid tissues, normalised for *ACTB* expression. GD, Graves' disease; TTN, toxic thyroid nodule and stTTN, corresponding normal thyroid tissue of same patient; CTN, cold thyroid nodule and stCTN, corresponding normal thyroid tissue of same patient; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma and ATC, anaplastic thyroid carcinoma (see Table 1).

Figure 2 (continued)

Figure 2 Immunodetection of *DEHAL1* protein expression in different thyroid pathologies. (A) Graves' disease, (B) toxic thyroid nodule, (C) surrounding tissue of toxic thyroid nodule, (D) cold thyroid nodule, (E) surrounding tissue of cold thyroid nodule, (F) follicular thyroid carcinoma, (G) papillary thyroid carcinoma, (H) anaplastic thyroid carcinoma, (I) medullary thyroid carcinoma (200× magnification).

different thyroid functional and/or differentiation states, we performed mRNA analyses of *DEHAL1* and its isoform *DEHAL1B* and studied *DEHAL1* protein expression by immunohistochemistry in a series of hyper- and hypofunctioning thyroid nodules, Graves' disease thyroids, normal thyroid tissues and different thyroid cancers.

DEHAL1 expression was altered in differentially functioning thyroid pathologies, whereby changes in mRNA and protein expression levels were largely concordant. The highest *DEHAL1* expression levels were detected in GD samples and toxic thyroid nodules, which is in agreement with stimulation of thyroid hormone production and as a 'by-process' of

Figure 3 Localisation of *DEHAL1* in papillary thyroid carcinomas. In PTCs with a conventional papillary appearance (A), diffuse and cytoplasmic *DEHAL1* is apparent, whereas in partially differentiated papillary thyroid carcinoma (B), *DEHAL1* protein expression is absent.

increased MIT/DIT deiodination. Thus, while upregulation of NIS in the hyperfunctional lesions will allow efficient iodine trapping, upregulation of *DEHAL1* will ensure efficient iodine recycling. Both mechanisms contribute towards a highly effective generation of thyroid hormones in thyrotoxicosis. Furthermore, the localisation of *DEHAL1* protein in GD thyroids and TTNs towards the apical pole is in line with *in vitro* data suggesting that *DEHAL* action takes place in close proximity to endoluminal TG uptake and proteolysis (4).

An increased, albeit much weaker (compared with the hyperfunctional states) *DEHAL1* mRNA expression was detected in CTN compared with their ST. Again, this could be linked to altered thyroid hormone synthesis, which is ineffective in CTN, primarily as a consequence of impaired iodide trapping (7, 10, 11). It is noteworthy that an increased expression of other components of the thyroid-hormone synthesising apparatus, e.g. TPO and thyroid oxidase, has recently been observed by differential proteome analysis of cold thyroid nodules (12). In addition, CTN and ST (as opposed to hyperfunctional lesions) exhibited a focal, rather than uniform *DEHAL1* expression pattern. *DEHAL1* staining was most prominent in small follicles, which are believed to be more actively involved in thyroid hormone turnover than the large colloid storing follicles (13, 14).

Additionally, we tried to elucidate the value of *DEHAL1* as a marker of thyroid differentiation. Prominent *DEHAL1* protein expression was observed in follicular thyroid carcinomas; however, in these cancers, *DEHAL1* expression was diffuse and there was no predominance of *DEHAL1* expression at the apical cell region. Diffuse cytoplasmic localisation was also observed in papillary thyroid cancers, albeit

with an overall weaker *DEHAL1* expression. In contrast, in partially differentiated cancers and undifferentiated thyroid cancers, protein staining was very faint or mostly absent (Figs 4A and B). A similar pattern in tumour-specific *DEHAL1* expression was also apparent on the mRNA level. These findings suggest a loss of *DEHAL1* with ongoing thyroid cell dedifferentiation.

Furthermore, we found a significantly higher expression of *DEHAL1B* variants in FTC and CTN. To date, there are no consistent data on the physiological relevance of the *DEHAL1B* isoform. Gnidehou *et al.* recently have cloned and characterised another *DEHAL1* isoform termed *DEHAL1C* (3). They show that the mRNA expression of *DEHAL1B* and *DEHAL1C* variants were lower in thyrocytes compared with *DEHAL1* and suggested that this is linked to a more rapid proteosomal degradation. However, the functional consequences regarding thyroid hormone metabolism are still unknown.

In summary, our study provides first evidence that *DEHAL1* might be a marker of differentiated thyroid function and that upregulation and predominant apical *DEHAL1* localisation occurs in the hyperfunctional states, while thyroid malignancy is associated with diffuse cytoplasmic localisation (differentiated FTC/PTC) or complete loss of *DEHAL1* expression (partially differentiated thyroid cancers and ATC). To follow up on the hypothesis of a functional relevance of altered intracellular *DEHAL1* localisation, further studies on the molecular regulation of *DEHAL1* expression and its isoforms are mandatory and will be crucial for the understanding of the physiological and a putative pathophysiological role of *DEHAL1* in the thyroid.

Figure 4 Semiquantitative analysis of *DEHAL1* protein expression in thyroid pathologies. *DEHAL1* staining was assessed by determining the percentage of positively stained thyrocytes/six randomly selected sections/slide (200 $\times$ magnification, duplicates). The degree of staining was quantified as follows: –, no staining; +, <30% staining; ++, 30–60% staining; +++, >60% staining. (A) Benign and malignant thyroid disease: Graves' disease (GD; $n=5$), toxic thyroid nodule (TTN; $n=11$), surrounding tissue of toxic thyroid nodule (stTTN; $n=11$), cold thyroid nodule (CTN; $n=16$), surrounding tissue of cold thyroid nodule (stCTN; $n=16$), follicular thyroid carcinoma (FTC; $n=8$), papillary thyroid carcinoma (PTC; $n=64$), partially differentiated thyroid carcinoma (PDTC; $n=22$), undifferentiated (anaplastic) thyroid carcinoma (ATC; $n=6$), medullary thyroid carcinoma (MTC; $n=5$). (B) Subgroup analysis for papillary thyroid carcinoma (PTC; $n=64$): follicular variant PTC ($n=17$), conventional (classic) PTC ($n=12$) and tall cell PTC ($n=34$).

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