Short Communication

Lack of complex I is associated with oncocytic thyroid tumours

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Oncocytic tumours are characterised by hyperproliferation of mitochondria. We immunohistochemically analysed all enzymes of the oxidative phosphorylation system in 19 oncocytic thyroid tumours. A specific lack of complex I was detected, which was expressed at <5% of the level determined in surrounding non-cancerous tissue.

Keywords: complex I deficiency; oncocytic thyroid tumour; respiratory chain

MATERIALS AND METHODS

Patients

Human oncocytic thyroid tumour (n = 19) and follicular thyroid adenoma (n = 4) specimens were obtained from the Department of Pathology, Paracelsus Private Medical University, Salzburg. The study was carried out in accordance with the guidelines of the local research ethics committee. Clinical parameters are summarised in Table 1.

Immunohistochemical staining and analysis

For immunohistochemical staining, the following antibodies were used: mouse monoclonal anti-complex I subunit NDUFS4 (1:1000; Abcam, Cambridge, UK), mouse monoclonal anti-complex II subunit 70kDa (1:3000; Mitosciences, Eugene, OR, USA), mouse monoclonal anti-complex III subunit core 2 (1:1500; Mitosciences), mouse monoclonal anti-complex IV subunit I (1:1000; Mitosciences), mouse monoclonal anti-complex V subunit-α (1:2000; Mitosciences) and mouse monoclonal anti-porin 31HL (1:3000; Mitosciences). All antibodies were diluted in Dako antibody diluent with background reducing components (Dako, Glostrup, Denmark).

Tissue sections (5 μm) were deparaffinised by three changes of xylene, rehydrated in three changes of absolute 2-propanol followed by heat-induced epitope retrieval in TE-T buffer (10 mM Tris pH 9.0, 1 mM EDTA, 0.05% Tween 20) for 40 min at 95°C and 20 min at room temperature. Sections were washed in distilled H2O and equilibrated with phosphate-buffered saline containing 0.5% Tween 20 (PBS-T pH 7.4). Staining was carried out using the Envision Detection System (Dako) according to the manufacturer’s instructions followed by visualisation with diaminobenzidine (DAB) for 10 min. Slides were counterstained with haematoxylin.

To quantify differences in expression levels between tumour tissue and the adjacent normal tissue, a scoring system for the staining intensity (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining) multiplied by the mean percentage of immunopositive cells per high power fields was used. Quantification was carried out independently by two different persons and mean values are displayed.

Statistical analysis

For statistical analysis, the Wilcoxon matched-pair signed-rank test was used for equality of distributions. The distribution of complex I and complex V were compared between normal tissue...
and the corresponding tumour tissue, respectively. The distribution of complex I was also compared to complex V in normal tissue.

**Table I** Clinical parameters of the patients with oncocytic thyroid tumours

| Parameter         | Determined | Values: mean (range) |
|-------------------|------------|----------------------|
| Age               | 19/19      | 55.6 years (27.6–83.8) |
| Gender            | 19/19      |                       |
| Male              | 3/19       |                       |
| Female            | 16/19      |                       |
| Diagnosis         | 19/19      |                       |
| Adenoma           | 17/19      |                       |
| Carcinoma         | 2/19       |                       |
| Tumour size       | 19/19      | 2.6 cm (1–5.3)        |
| Endocrine situation| 18/19      |                       |
| Hypothyroid       | 1/18       |                       |
| Euthyroid         | 14/18      |                       |
| Hyperthyroid      | 3/18       |                       |
| Goitre            | 15/19      |                       |
| Yes               | 15/15      |                       |
| No                | 0/15       |                       |
| Immune thyroiditis| 16/19      |                       |
| Yes               | 4/16       |                       |
| No                | 12/16      |                       |

**Analysis of mtDNA**

Two 10 μm sections were collected and deparaffinised by two changes of xylene and absolute ethanol. After evaporation of ethanol, tissues were treated with 200 μl of PCRK (2 mg ml⁻¹ Proteinase K, 75 mM Tris-HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween 20) at 60 °C for 24 h followed by 95 °C for 10 min. Sequence analysis of the mtDNA was carried out as described previously (Mayr et al, 2008).

**RESULTS**

**Immunohistochemical staining**

Oncocytic thyroid tumour cells were negative or greatly reduced for the complex I subunit, NDUFS4, when compared with the surrounding non-cancerous tissue in all investigated specimens (Figure 1). Immunopositivity for complex II subunit 70 kDa, complex III subunit core 2, complex IV subunit I, complex V subunit-a and the mitochondrial membrane protein porin was increased in all tumour samples compared with adjacent normal tissue (Figure 1). These changes were detected in all 17 oncocytic adenomas and the two cases of oncocytic thyroid carcinomas. Follicular thyroid adenomas showed substantial staining for all respiratory chain enzymes and porin (Supplementary Figure 1).
Molecular Diagnostics

1436

eight cases, no potentially pathogenic mutation was found. Conserved domains (3392G and three cases of mutations causing amino-acid changes in highly conserved domains (10477delT/10952_10953insC, 13235_13236insT, 14339_14340insA, 14603_14604insT), one case of a nonsense mutation (12539G -> T)). One third of the samples, where 33% had frameshift mutations, 6% stop mutations and 17% potentially pathogenic point mutations. We cannot exclude mutations in the protein-coding domains of the 39 nuclear-encoded subunits. An earlier study has already reported heterozygous mutations in GRIM-19 (Maximo et al, 2005), a subunit of mitochondrial complex I, which is also necessary for assembly.

DISCUSSION

Here, we provide strong evidence for an association between specific loss of respiratory chain complex I and oncocytic thyroid tumours. Mutations of mtDNA had previously been identified in oncocytic thyroid tumours; 26.7% of specimens showed disruptive mutations. Additional 26.7% had potentially deleterious missense mutations in one of the seven mitochondrial genes coding for subunits of complex I (Gasperre et al, 2007). Similar results were obtained in our samples, where 33% had frameshift mutations, 6% stop mutations and 17% potentially pathogenic point mutations. We cannot exclude mutations in one of the 39 nuclear-encoded subunits. An earlier study has already reported heterozygous mutations in GRIM-19 (Maximo et al, 2005), a subunit of mitochondrial complex I, which is also necessary for assembly.

Loss of complex I in oncocytic thyroid tumours

FA Zimmermann et al

On account of the heterogeneous composition of thyroid tumours, it is difficult to prepare pure tumour homogenates for enzyme measurements or western blot analysis. Gasparre et al (2007) were, therefore, not able to report biochemical results of their specimens. It is known from cell culture studies (Hofhaus and Attardi, 1993) and from patients with complex I defects (Ugalde et al, 2004a) that severe mutations in different subunits of complex I result in reduced stability or incomplete assembly of the enzyme complex. Moreover, non-mutant subunits of complex I exhibit variable stability (Ugalde et al, 2004b). For example, the NDUF4 subunit is particularly unstable and its stability depends on the presence of the preassembled complex I. Because of this instability of unassembled NDUF4, we used immunohistochemical staining with an NDUF4 antibody as a measure of complex I content.

Complex I is an integral component of different apoptotic pathways (Ricci et al, 2004; Martinvalet et al, 2008). Thus, deficiency of complex I could play a role in tumour formation by interrupting apoptotic pathways. Besides its function as part of the respiratory chain, complex I can be transformed to a potent enzyme of reactive oxygen species (ROS) formation. It was shown that the NDUF5 subunit of complex I can be proteolytically cleaved by caspase-3, which results in a truncated enzyme complex disposed for ROS production (Ricci et al, 2004). In a second, caspase-independent apoptotic pathway granzyme A specifically cleaves subunit NDUF5 of complex I. This again results in increased generation of ROS, disruption of the mitochondrial transmembrane potential and cell death (Martinvalet et al, 2008). Lack of complex I, as identified in oncocytic thyroid tumours, may, therefore, prevent tumour cells from undergoing apoptosis through these pathways.

The clinical parameters shown in Table 1 are typical for the diagnosis oncocytoma and the complex I deficiency was independent of age, gender, tumour size, histology and endocrine situation. All investigated thyroid oncocytomas showed complex I deficiency and most had a benign clinical course; however, 2 of the 19 tumours in this study were carcinomas. Renal oncocytoma, another type of oncocytic tumour with a benign course, was recently found to be associated with complex I deficiency (Gasparre et al, 2008; Mayr et al, 2008). The oncocytic tumour cell line XTC.UC1, which is derived from a metastasis of a Hürthle cell carcinoma, shows both an insertion mutation in the gene encoding ND1 subunit of complex I and a reduction in complex I activity (Bonora et al, 2006). Ishikawa et al (2008) reported complex I deficiency caused by mutations in the ND6 gene of complex I in non-oncocytic mouse lung tumours. Interestingly, these mutations increased the metastatic potential of the tumours (Ishikawa et al, 2008), whereas complex I deficiency in oxyphilic tumours does not seem to be associated with metastasis. For that reason, complex I deficiency is not considered as a specific feature of benign oncocytic tumours.

To establish that isolated complex I deficiency is a characteristic of oncocytic thyroid tumours, but not a general feature of thyroid tumours, we analysed four follicular thyroid adenoma samples. In contrary to oncocytomas, follicular thyroid adenomas displayed no significant reduction of respiratory chain enzymes.

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