Mitochondrial DNA mutations in renal cell carcinomas revealed no general impact on energy metabolism

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Previously, renal cell carcinoma tissues were reported to display a markedly reduced activity of components of the respiratory chain. To elucidate a possible relationship between tumourigenesis and alterations of oxidative phosphorylation, we screened for mutations of the mitochondrial DNA (mtDNA) in renal carcinoma tissues and patient-matched normal kidney cortex. Seven of the 15 samples investigated revealed at least one somatic heteroplasmic mutation as determined by denaturing HPLC analysis (DHPLC). No homoplasmic somatic mtDNA mutations were observed. Actually, half of the mutations presented a level of heteroplasmy below 25%, which could be easily overlooked by automated sequence analysis. The somatic mutations included four known D-loop mutations, four so far unreported mutations in ribosomal genes, one synonymous change in the ND4 gene and four nonsynonymous base changes in the ND2, COI, NDS and ND4L genes. One renal cell carcinoma tissue showed a somatic A3243G mutation, which is a known frequent cause of MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis, stroke-like episode) and specific compensatory alterations of enzyme activities of the respiratory chain in the tumour tissue. No difference between histopathology and clinical progression compared to the other tumour tissues was observed. In conclusion, the low abundance as well as the frequently observed low level of heteroplasmy of somatic mtDNA mutations indicates that the decreased aerobic energy capacity in tumour tissue seems to be mediated by a general nuclear regulated mechanism.

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Renal cell carcinoma is the most common malignancy arising in the adult kidney. Already five decades ago it was shown that the vast majority of tumours display a high rate of glycolysis under aerobic conditions (Warburg, 1956). Accordingly, in renal cell carcinomas, an increase of proteins involved in major steps of the glycolytic pathway and a decrease of the gluconeogenic reactions was observed (Faure Vigny et al., 1996; Unwin et al., 2003). Furthermore, a depletion of the activity of several mitochondrial enzymes was demonstrated (Faure Vigny et al., 1996; Unwin et al., 2003; Meierhofer et al., 2004). Human solid tumours endure profound hypoxia, which indicates that adaptation to hypoxic conditions is a crucial step in tumour progression. Recently, it was postulated that deficiency of the von Hippel Lindau (VHL) protein, which is observed in most renal carcinomas, could be one of the factors responsible for downregulation of the biogenesis of complexes of the oxidative phosphorylation (OXPHOS) (Shiao et al., 2000; Hervouet et al., 2005).

Since some subunits of the OXPHOS are encoded by mitochondrial DNA (mtDNA), alterations of mtDNA may influence OXPHOS activity. Mitochondrial DNA has a high mutation rate due to the damage produced by free radicals, the lack of protective action by histones and the limited capacity of repair of the mtDNA (Brown et al., 1979; Pettepher et al., 1991). The mutation rate of mtDNA has been reported to be as much as two orders of magnitude greater than that of nuclear DNA (Khrapko et al., 1997). A mutation in mtDNA expands either partially (heteroplasmy) or totally replaces all mtDNA (homoplasmy). However, it is still unclear how mutated mtDNA expands in cells.

Recently, a high incidence of specific mtDNA alterations has been reported for gastric (Maximo et al., 2001; Wu et al., 2005), prostate (Jeronimo et al., 2001; Petros et al., 2005), pancreatic (Jones et al., 2001), skin (Giral-Rosa et al., 2005), colorectal (Polyak et al., 1998; Hibi et al., 2001a; Lievre et al., 2005), urinary bladder (Fliss et al., 2000), thyroid (Yeh et al., 2000), oesophageal (Hibi et al., 2001b; Kumimoto et al., 2004), liver (Nishikawa et al., 2001), breast (Richard et al., 2000; Tan et al., 2002; Zhu et al., 2005), uterine cancers (Pejovic et al., 2004) as well as chromophobe renal cell carcinoma (Nagy et al., 2002). Of all mtDNA mutations reported in cancer tissues, only a few are known to be of pathological relevance as shown for patients with disorders of the mitochondrial energy metabolism. For example, a G5521A mutation, which is known to cause a late onset mitochondrial myopathy (Silvestri et al., 1998), was detected in a lung cancer tissue (Fliss et al., 2000). A mutation (G13708A) reported in a lung cancer tissue (Parrella et al., 2001)
and a mutation typical for mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) was detected in a colon cancer sample (Lorenc et al., 2003). As would be anticipated, these patients, who all were carrying only tumour-specific pathogenic mtDNA mutations, did not show clinical signs of mtDNA-related disease. Recently, it was shown that the introduction of a pathogenic mtDNA mutations, for example, in the mitochondrial-encoded ATPase 6 gene (MTATP6), can lead to declined respiration and accelerated growth of tumour cells via inhibition of apoptosis (Petros et al., 2005; Shidara et al., 2005).

To determine whether the downregulation of the enzyme activities reported in renal carcinoma is associated with mtDNA mutations, we screened the entire mtDNA of 15 paired tumour and the corresponding normal kidney samples by denaturing HPLC analysis (DHPLC) analysis. Occurrence and type of somatic mtDNA mutations were compared to the enzymatic activity of respiratory chain complexes.

MATERIALS AND METHODS

Patients

Tumour and the corresponding healthy cortex tissue from 15 patients were obtained by nephrectomy at the Department of Urology, Salzburg as previously reported (Meierhofer et al., 2004). The tumour classification was performed according to Storkel et al. (1997). DNA was isolated by proteinase K digestion followed by phenol/chloroform extraction.

PCR amplification of entire mitochondrial genome and mutation analysis

The whole human mtDNA of all 15 patients was amplified using 48 overlapping PCR fragments. Mutation detection by DHPLC was performed as reported previously (Meierhofer et al., 2005). As estimated from the areas under the peaks of the DHPLC chromatogram, the degree of heteroplasmy was divided into lower 25% (< 25%), between 25 and 75% (25 – 75%) and over 75% (> 75%). All samples showing a homo- and heteroduplex peak of the same extent in DHPLC analysis were sequenced directly. Heteroplasmic peaks lower than the homoplasmic peak were manually collected and reamplified as previously reported (Meierhofer et al., 2005).

Mutations were analysed with the Beckman software investigator (Fullerton, CA, USA). Exact positions of mutations and amino-acid changes were defined with the mitoAnalyser tool (MitoAnalyzer, 2000), using the mtDNA genbank sequence J01415.1 as reference.

Enzyme measurements

The following enzyme activities: citrate synthase, complex II, complex IV and oligomycin-sensitive ATPase activity of complex V were determined and reported previously (Meierhofer et al., 2004). In addition, complex I activity was measured according to Rustin et al. (1994).

Western blot analysis of the VHL tumour suppressor protein

After separation of the 600 g homogenate (Meierhofer et al., 2004), 18 μg protein per lane were loaded on a 10% polyacrylamide gel and Western blot analysis was performed according to Berger et al. (2003). The following antibodies were used: mouse monoclonal antibody against VHL protein (Cat# 556347; BD Bioscience, Palo Alto, CA, USA; 1 μg/ml), alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (Dako, Golstrup, Denmark; 1: 5000).

RESULTS

MtDNA mutations

The mutation screening of the entire mtDNA of 15 primary renal carcinoma and matched control kidney cortex tissues was carried out by DHPLC analysis. Five matched tumour/kidney pairs showed one to two heteroplasmatic mutations (six D-loop, one 16s rRNA and one COI) of the mtDNA with no difference of the degree of heteroplasmy in the two tissues (Table 1). Interestingly, in two patients, a shift in heteroplasmy from the kidney to the corresponding tumour tissue was observed (cases 11, 12; Table 1, Figure 1). Loss of low-level heteroplasmy present in normal cortex tissue was observed in cases 4 (310 C insertion; A8483G) and 14 (T72C). Only three conventional renal carcinomas with grades 1, 2 and 4 carried neither a somatic nor a heteroplasmic mtDNA mutation.

Table 1 Nonsomatic heteroplasmies in matched tissue pairs

| Case | Tumour type | Grade | Nucleotide position | Heteroplasmya in kidney/tumour (%) | Gene | Amino acid | Notes |
|------|-------------|-------|---------------------|-----------------------------------|------|------------|-------|
| 6    | Papillary RC | 2     | A16206G             | > 75                              | D-loop | d          |       |
| 7    | Sarcomatoid RC | 4     | C16348T             | > 75                              | D-loop | d          |       |
| 11   | Conv. RCb   | 3     | T16092C             | from 40 to > 95                   | D-loop | d          |       |
| 12   | Conv. RC    | 2     | T8750C              | from 50 to 80                     | ATP6  | L75S       | d     |
| 13   | Conv. RC    | 1/2   | T1809C              | > 75                              | ATP6  | L75S       | d     |
| 14   | Papillary RC | 1     | G6260A              | 25 – 75                           | COI   | syn        | c     |
| 15   | Conv. RC    | 1     | T16093C             | 25 – 75                           | D-loop | d          | c     |

a Estimated by denaturing HPLC analysis. b Conv. = conventional; RC = renal carcinoma; syn = synonymous. c Previously reported polymorphism (MITOMAP database). d = Novel mutation.

Determination of mutational load of the A3243G mutation

A PCR fragment was amplified with the following primers: forward, 5’-CTCCGTGAAAGGATACAAGA-3’; reverse, 5’-AGGA GTAGGAGTTGCCCAG-3’. Then, digestion with HaeIII, which digests only the mutation-specific restriction side 3243G and one control restriction side on each side of the PCR fragment, was performed. The percentage of mutational load was detected by densitometry of ethidium-bromide-stained agarose gels (Uziel et al., 1997; van den Bosch et al., 2004).
A3243G mutation revealed an 89% mutational load (Figure 2F). The corresponding kidney tissue was unaffected.

Furthermore, the known MELAS suppressor G12300A mutation (El Meziane et al., 1998) was not present in the tumour sample with the A3243G mutation.

Enzyme activities

Biochemical analysis of OXPHOS and Krebs-cycle enzyme activities of 37 renal carcinoma tissues were reported in our previous study (Meierhofer et al., 2004), which included the 15 patients presented here (Table 3). A total of 14 renal carcinoma tumour tissues, investigated in this study, showed a depletion of the mtDNA content (46%) combined with a decreased activity of complex I (16%), complex II (23%), complex IV (29%), oligomycin-sensitive ATPase activity of complex V (25%) and citrate synthase (72%) compared to matched normal cortex tissues (Table 3) (Meierhofer et al., 2004).

This is in clear contrast to the tumour tissue with the A3243G mutation, which showed an upregulation of the mtDNA content (170%) and increased enzyme activities of complex II (138%), oligomycin-sensitive ATPase activity of complex V (1530%) and citrate synthase (575%) and a downregulation of the mitochondrial- and nuclear-encoded enzyme activities of complex I (22%) and complex IV (78%) related to the matching control tissue (Table 3).

One tumour tissue displayed only an elevated level of citrate synthase (300%). As expected, no pathogenic mutation was found in the mtDNA of this sample.

Western blot analysis of the VHL tumour suppressor protein

The von Hippel-Lindau tumour suppressor protein (VHL) is frequently absent in renal carcinoma tissues. A recent study has shown that VHL deficiency is one of the factors responsible for downregulation of the biogenesis of OXPHOS complexes in renal carcinoma (Hervouet et al., 2005). To exclude that the altered enzyme activities of the OXPHOS complex in the tumour tissue with the A3243G mutation are based on the presence of the VHL protein, we tested tumour tissues by Western blot analysis for VHL content. In the renal carcinoma tissue harbouring the A3243G mutation as well as in the two other renal carcinoma tissues investigated, lack of VHL protein was observed (Figure 3).

DISCUSSION

In the present study, we evaluated the influence of mtDNA mutations on the OXPHOS capacity of renal carcinoma tissues. The mtDNA was more frequently mutated in renal carcinoma than in kidney cortex tissues. This is in line with somatic mtDNA mutations reported in a wide variety of human neoplasias (Fliss et al., 2000; Jones et al., 2001; Nagy et al., 2002; Tan et al., 2002). Somatic mutations in coding regions of the mtDNA in tumour tissues are either silent or mostly nonpathogenic polymorphisms. Only a few pathogenic mtDNA mutations have been reported (Brown et al., 1992; Parrella et al., 2001; Lorenc et al., 2003). However, no biochemical analysis has been performed to evaluate the consequences of these mutations in the tumour tissues. Here, we show the first combined genetic and biochemical analysis of a mitochondrial A3243G mutation in a tumour tissue. The renal carcinoma tissue with the somatic A3243G mutation showed a compensatory upregulation of enzyme complexes, which are only nuclear encoded. This is in clear contrast to the coordinated downregulation of all components necessary for mitochondrial energy metabolism, which we found in 92% of all renal carcinoma tissues irrespective of tumour stage and progression (Meierhofer et al., 2004).
Table 2  Gain of somatic mutations of the entire mitochondrial DNA genome in renal carcinoma tissues

| Case | Tumour type | Grade | Nucleotide position | Heteroplasmy* in tumour (%) | Gene | Amino acid | Notes |
|------|-------------|-------|---------------------|------------------------------|------|------------|-------|
| 1    | Conv. RC   | 2/3   | C338T               | <25                          | D-loop | c          |       |
| 2    | Conv. RC   | 3     | G1578G              | <25                          | 12S rRNA | d          |       |
| 3    | Conv. RC   | 2     | G11007A             | <25                          | ND4   | syn        |       |
| 4    | Conv. RC   | 3     | G15784A             | <25                          | ND2   | A39T       | d     |
| 5    | Papillary RC | 1   | T204C               | >75                          | D-loop | c          |       |
| 6    | Papillary RC | 2   | G169A               | >75                          | COI   | E507G      | d     |
| 7    | Sarkomatoid RC | 4  | G15100A             | >75                          | rRNA  | tRNA LEU(UUR) | MELAS |
| 8    | Papillary RC | 1   | T10579C             | >75                          | ND5   | D58E       | d     |
| 9    | Papillary RC | 2   | C1566T              | >75                          | 16S rRNA | d          |       |
| 10   | Papillary RC | 3   | T10579C             | >75                          | 12S rRNA | d          |       |
| 11   | Papillary RC | 4   | T10579C             | >75                          | ND4L  | M37T       | d     |
| 12   | Papillary RC | 5   | T10579C             | >75                          | D-loop | c          |       |

*Estimated by denaturating HPLC analysis. **Conv. = conventional; RC = renal carcinoma; syn = synonymous. *Previously reported polymorphism (MITOMAP database). **Novel mutation.

Figure 2  Mitochondrial DNA analysis of the renal carcinoma with the somatic A3243G mutation. Denaturating HPLC analysis of a PCR fragment (3079–3505) of case 4 at 58°C oven temperature (A–C). The kidney tissue shows a single homoplasmic peak (A), the corresponding renal carcinoma tissue a heteroplasmy over 90% (B) and the mixture of denaturated and reannealed PCR product of kidney and the corresponding tumour tissue resulted in one hetero- and homoduplex peak of the similar height (C). Sequence analysis of the PCR product indicated the wild-type 3243A variant in the unaffected kidney tissue (D), and the 3243G mutation in the corresponding carcinoma tissue (E), as indicated by arrows. Agarose gel analysis of a restriction digestion of a PCR fragment (3118–3332) with HaeIII, which specifically recognizes the 3243G mutation, and two control sites within the PCR fragment yielding two small fragments (F): undigested full-length PCR fragment of 215 base pairs (lane 1); kidney tissue resulting in a 169-base pair fragment (lane 2); carcinoma tissue resulting in a weak 169-base pair fragment of the residual wild-type 3243A variant as well as 72- and 97-base pair fragments, indicating the 3243G mutation (lane 3); 100 bp molecular weight marker (lane 4). R = G + A.
The rate of DNA replication by modifying the binding affinity of significant trans-activating factors. In the renal carcinomas presented here, only four out of the 14 somatic mutations affected the D-loop. This cannot explain the decrease of the mtDNA content observed in the majority of renal carcinomas (Simonnet et al., 2002; Meierhofer et al., 2004). In agreement with other studies, either G to A or T to C transitions were observed, which is consistent with the mutagenic spectra of oxidative damage (Lee et al., 2004).

In half of the somatic mutations, the level of heteroplasmy was between 1 and 25%. Pheno- typic manifestation of a genetic defect of the mtDNA occurs only if a threshold level is exceeded. Although the phenotypic threshold depends on the type of mutation and tissue, it has been shown that a heteroplasmy at least over 60% is necessary to show effects on the enzyme activity of respiratory chain complexes (Rossignol et al., 2003). Therefore, a primary role of the low heteroplasmic mutations in downregulation of OXPHOS activity and renal carcinogenesis is unlikely. Accumulation of mtDNA mutations in tumours can be explained without selection (Coller et al., 2001). Heteroplasmic mutations might have accumulated in cancer cells by random drift of a pre-existing heteroplasm (Nekhaeva et al., 2002). This random drift will also explain the observed shift of the degree of heteroplasmy of a mixture of mutated and wild-type mtDNA already detectable in tumour progenitor cells.

In conclusion, the low frequency as well as the low level of heteroplasmy of the somatic mtDNA mutations in renal carcinomas does not indicate a major contribution of these alterations in tumour development. Furthermore, the downregulation of the mitochondrial energy metabolism observed in renal carcinomas cannot be explained by the presence of mtDNA mutations. Even, the case carrying the A3243G mutation exhibits normal tumour histology and low overall oxidative capacity. More likely, a general nuclear-encoded mechanism results in the adaptation of the aerobic energy metabolism in renal carcinoma.

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