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Contribution of proteomics to the molecular analysis of renal cell carcinoma with an emphasis on manganese superoxide dismutase

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Abstract. Renal cell carcinoma (RCC) originates in the renal cortex. It accounts for 2—3 percent of all cancers occurring in adults and it is characterised by lack of early clinical manifestations, unpredictable outcome, and absence of effective treatment modalities except early surgery. RCC comprises a heterogeneous group of tumours with various molecular and cytogenetic abnormalities and different histological features as cell types and tumour architecture. Molecular genetic and proteomic tools led to the discovery of potential diagnostic prognostic and therapeutic biomarkers of RCC. In this review we discuss recent developments in understanding genotype-phenotype relationships, with attention to manganese superoxide dismutase, a mitochondrial enzyme related to the redox cycle which affects various regulatory functions of cells. The expression of this protein has been evaluated in numerous human tumour types including RCC, and post-translational modifications are being investigated.

Keywords. Two-dimensional electrophoresis / Renal cell carcinoma / Manganese superoxide dismutase / Post-transitional modifications / Review

Abbreviations. IFN, interferon; IL, interleukin; Mn-SOD, manganese superoxide dismutase; RCC, renal cell carcinoma

Introduction

Renal cell carcinomas (RCC) are a heterogeneous group of tumours representing about 3% of all adults cancers in Western countries (1). RCCs originating in the renal cortex account for 80—85% of primary malignancies of the kidney. In most of the cases, RCCs occur in sporadic form and only a small fraction shows a recognisable hereditary pattern. Since small, localised tumours rarely produce symptoms and the diagnosis of RCC is often delayed until the disease is well advanced. In addition, compared with other urological cancers, RCCs are associated with a high potential of metastases (2). As the majority of tumours are derived from epithelial ceils, RCCs are often composed of a variety of different cell types with different degrees of differentiation (3). Histological features distinguish clear cell, the most frequent type, from chromophilic, granular, chromophobic, oncocytic, and mixed cell types (Fig. 1) (4). RCCs are also classified according to their growth patterns. Non-papillary tumours are characterised by abnormalities of 3p chromosome, while papillary tumours do not show these abnormalities (5).

Neither chemotherapy nor radiotherapy are effective therapy for RCC (6). Nephrectomy remains the cornerstone of treatment for this tumour type, even though immunotherapeutic and genetic approaches are providing further options (6-8). Currently there are no validated markers for either detection or monitoring of RCC evolution and treatment. However, numerous efforts are being carried out using molecular genetic, cytogenetic, immunohistochemical analyses and/or proteomic approaches to further characterize RCC.

Diagnostic and prognostic biomarkers for RCC

So far, no single protein marker has been proven useful in screening for asymptomatic disease, for dia-

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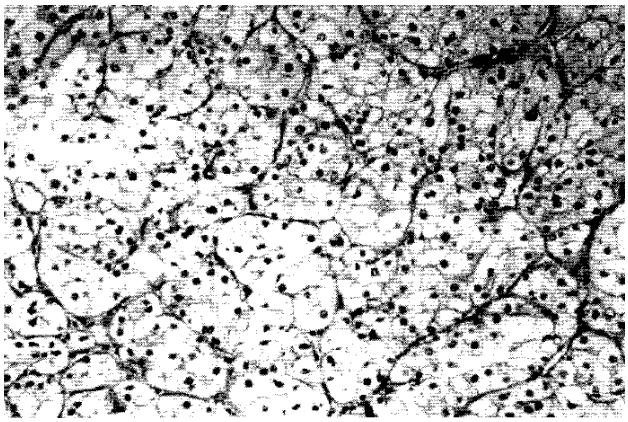
Figure 1. Photomicrograph of renal cell carcinoma haematoxylin-eosin stained exhibiting mixture of clear and granular cell types. Granular cells show eosinophilic cytoplasm in contrast to cytoplasm of clear cells.



gnosis, or for staging and monitoring of RCC treatment. The identification of diagnostic and prognostic markers including proliferation markers, karyometric analyses, oncogenes, proteases and cell adhesion molecules remains an area of intense investigation.

Serum levels of erythropoietin, ferritin, neuron specific enolase and transforming growth factor, were assayed and have not been found to be of any utility in clinical management of RCC patients (9-12). The expression of the tumour proliferation marker Ki-S5 was found to correlate with survival. This is in contrast to reported findings regarding the expression of another proliferation marker, the Ki-67 antigen, and of the tumour suppressor p53 as prognostic indicators in RCC (13-15). The p53 gene does not play a major role in development of RCC, whereas in early events of tumorigenesis high expression of c-neu and c-fos oncogenes, and proto-oncogene bcl-2, inhibitor of apoptosis, is detected (16-17). This pattern of gene expression may explain in part the relative resistance of RCC to chemotherapeutic agents and radiotherapy. Highly significant associations between molecular events and cytomorphological subtypes of RCC were detected using immunohistochemical tissue staining. The results suggested a role for c-met and epidermal growth factor receptor (EGFR) in RCC morphogenesis and for the von Hippel-Lindau (VHL) tumour suppressor gene in the clear cell subtype. There is a relationship between c-met overexpression and an RCC chromophilic subtype with papillary growth pattern, and between overexpression of EGFR and an RCC clear cell subtype with a compact growth pattern (18). Beside, c-met mutations were observed on 75% of papillary RCC patients with multiple bilateral lesions (19).

Recently, the TIAM1 protein gene product has been identified. This 177 kDa protein activates RHO-like proteins and connects extracellular signals to cytoskeletal proteins affecting invasion in human tumours. Expression of the mutated TIAM1 gene in RCC was shown to be inversely correlated with invasion, suggesting a role for this gene in progression of human RCC (20). lnvasion of the renal vein plays an important prognostic significance. Studies of cell-cell adhesion molecules associated with tumour invasiveness highlighted also a significant correlation between catenin down-expression in RCC tissue and poor survival of patients, whereas E-cadherin and catenin p120cas expression were shown not to be prognostic indicators (21). Differential expression of cell-adhesion molecules a-2, a-5, and a-6 integrins, E-cadherin, neural adhesion molecule and CD44 showed a correlation with histological type and tumour grade, particularly strong expression of integrins seemed to be correlated with invasion of RCC cells (22-23).

Genomic aspects of renal cell carcinoma

An accumulation of genetic events may be responsible for renal tumorigenesis. However the genetic changes that occur are not well understood at the molecular level. The karyotypes of RCC are extremely complex and involve several chromosomes (24-25). The most consistent and recurrent abnormalities in familial and sporadic nonpapillary RCCs are deletions or rearrangements involving chromosome 3p, unbalanced translocations between 3p and 5q leading to loss of 3p1-pter, and duplication of 5q22-qter (5). Loss of heterozygosity analyses revealed that at least three putative tumour suppressor genes are located on chromosome 3p, at 3p14, 3p21.3 and 3p25 loci (26). The locus at 3p25 contains the VHL tumour suppressor gene, involved in the regulation of vascular endothelial growth factor (VEGF). Somatic mutations in the VHL gene are associated with sporadic RCC, especially clear cell types and nonpapillary RCC, in addition to the association between germ-line mutations and hereditary VHL syndrome (27). Mutations inactivating the VHL gene product (pVHL) are due to single base pair changes (28). Lack of a gene product is due to either deletion or DNA hypermethylation (29-39). The pVHL was identified as a negative regulator of the cell cycle, and implicated in formation of extracellular matrix, and in regulation of extracellular pH. However, different results related to VHL gene mutations and VHL mutation/hypermethylation frequencies were found in various investigations, perhaps related to tumour stage (28, 31-32). The familial form, associated with predisposition to an early-onset of multicentric RCC, was also characterised by balanced translocations t(3;8)(p21;q24) (33-34). Loss of heterozygosity for the wnt-5a gene mapped to chromosome 3p14-21, was reported to affect the suppression of growth and repression of telomerase activity, and was described in nonpapillary RCC (35). Besides, in region 3p21.1 is located the TU3A gene that codes for DRR1 protein found to be down

regulated in RCC (36). Finally, in region 3p26.2 is located the OGG1 gene that codes for DNAglycosylase/APlyase protein that was found to be drastically reduced in RCC with a strong impairment in its DNA repair capacity (37).

In advanced stages of sporadic RCC various changes involving chromosomes 5q, 6q, 7p, 8p, 9p, 13q, 14q, 17p, 22q, were observed including deletion, translocation, trisomy or more copies of chromosomes and loss of Xq, and Y (38-39). Cytogenetic studies revealed that numerous genes related to cell growth or proliferation, including platelet derived growth factor (PDGFRB), macrophage colony-stimulating factor-1 receptor (CSF1 R), fibroblast growth factor acidic (FGF1), interleukin-9 (ll-9), ras-specific nucleotide exchange factor (CDC25) and a phosphatase involved in the regulation of cell cycle progression (CDC25C), are located in the smallest region of overlap for increased chromosome 5q copy number, suggesting a gene-dosage mechanism in tumorigenesis. Currently, a strong correlation between chromosome 5q alterations and a high level of cytokine secretion was observed in addition to the association between increased chromosome 7 copy number, and interieukin-6 (IL-6) secretion (40-41). c-met gene expression was found to be increased in high proportion of RCC due to chromosome 7 trisomy. DNA methylation is an important mechanism that controls gene expression methylation. Hypomethylation of specific CpG island regulatory regions near the promoter was associated with the activation of the MN/CA9 gene mapped to chromosome 17q (42). The MN/CA9 gene product belongs to a family of zinc metalloenzymes normally expressed in the cytoplasm of gastric mucosa and large bile duct cells. It was localised on the surface and in the cytoplasm of RCC cells, and it was not detected in normal kidney (43). The potential utility of MN/CA9 gene product as RCC marker was demonstrated in a preliminary study by reverse transcriptase (RT)-PCR assay. This method was described as capable of detecting circulating cells expressing the MN/CA9 mRNA in the blood of patients with metastatic RCC (44). Similar results were obtained by flow cytometric analysis of MN/CA9 antigen from RCC tissue (45), suggesting its possible use as an antigen for radioimmunotherapy (46). As for most solid tumours, the RCC studies described are consistent with the evidence that RCC development occurs through a myriad of events involving different chromosomes and numerous genes.

Proteomic investigation of renal cell carcinoma

The sum of molecular, histological and clinical RCC studies suggests the need to investigate RCC by alternative approaches. Proteomic technologies can be applied to human cancer to analyse simultaneously the expression of multiple genes for changes at the protein level resulting from genomic changes, alternative splicing and post-transitional modification (47). Solid tumour tissues are a complex system where different cell types and proteins are present, including extracellular matrix proteins, serum proteins, blood vessels and blood cells, mesenchymal, endothelial, epithelial cells, as well as necrotic material. Since it is difficult to obtain purified epithelial cells from the mixture of histological samples for protein extracts, the number of reports in the literature describing the proteomic approach to investigate RCC is low . Some relied on RCC cell lines (49-51), and others used RCC tissue samples (52-53). Two-dimensional electrophoresis is a powerful means to separate and identify simultaneously thousands of polypeptides. A crucial step is the separation and partial purification of epithelial cells to obtain reproducible patterns for analysis of differences between normal and tumour tissues. Cell lines and primary cell cultures can produce *in vitro* artefacts generating heterogeneous results when compared to tissue (54), so protein extracts from tissues are needed. Heterogeneity of RCC cell lines was also revealed using 2-DE, highlighting different levels in the expression of proliferating cell nuclear antigen and glutathione-S-transferase (50). Specific antibodies to epithelial antigens bound to magnetic beads can be used to improve separation of epithelial cells (55-56), but laser capture microdissection is a more promising technique to resolve the problem of contamination and mixture of cell types (57). In addition, to improve the RCC protein characterization by 2-D PAGE, Starita-Geribaidi et *aI.* (58) described a protocol to analyse specific subcellular fractions such as basolateral membranes containing protease SP22OK, a potential RCC marker.

The 2-DE patterns of whole renal and RCC tissues revealed a number of polypeptides associated with RCC (52, 56). Four polypeptides, ubiquinol cytochrome *c* reductase, NADH-ubiquinone oxido-reductase complex I, and two isoforms of plasma giutathione peroxidase, were not detected in RCC, and three monomeric and two multimeric isoforms of manganese superoxide dismutase (Mn-SOD) were not present in normal kidney tissue. Moreover, the transitionally controlled tumour protein (TCTP), a growth related protein with likely housekeeping function, was ubiquitously detected in several healthy and tumoral cells but not in RCC (59). RCC tissue was also analysed to study the expression of human leucocyte antigen (HLA) class I by one-dimensionai IEF. The results, when analysed on the basis of tumour stage and data of microcytotoxicity assay, suggested that the loss of HLA expression is correlated with advanced stages (60). One-dimensbonal Western blot analysis was used to investigate cytokeratin expression and revealed similar phe-

notypic patterns between RCC and hepatocellular carcinoma tissues, as observed by immunohistochemical stain (61). Unfortunately, in the majority of cases, preliminary promising results of protein expression of RCC tissue were not validated with follow-up data (62). The combination of 2-DE with capillary electrophoresis or HPLC and with MS was utilized to define biochemical pathways of IFN-y and IL-4 action in RCC for their application to immunotherapy. Five proteins were increased after cytokine-induction including tropomyosin, heat shock protein 27, manganese superoxide dismutase, glutathione S-transferase pi and protein kinase C inhibitor I (51). These data illustrate that in response to just one of many physiological or pathological parameters, several proteins can be modified, highlighting the complexity of protein interactions and post-transitional modifications.

Manganese superoxide dismutase

Genetic, structure and function

Mn-SOD is a potent scavenger of superoxide radicals produced by oxidative phosphorylation into O_2 and H_2O_2 and plays a central role in the defence against oxidative stress. Mn-SOD is nuclear-encoded, synthesised in the cytosol and post-translationally modified by truncation of 24 amino acids for transport into mitochondria (63). There are three other distinct monomeric forms that are widespread in nature: the copper/zinc (Cu/Zn-SOD) found in the cytosol, the iron (Fe-SOD) isozyme present in the chloroplasts of higher plants (64), with an amino terminal targeting sequence for their respective subcellular compartments, and the extracellular (EC-SOD) glycosylated that is strongly homologous to Cu/Zn-SOD (65).

The active form of Mn-SOD is homotetrameric (66). The monomers fold into two domains with the N-terminal domain shaped of two long antiparallel a-helices, and C-terminal domain of a central b-sheet formed by three antiparallel b-strands with 4-6 surrounding a-heIices. The monomer contains a single active site binding one manganese atom that cycles between oxidised and reduced states. Two histidines from each of the N-termmal helices, and one histidine and aspartate from the loops in the C-terminal domain link the manganese atom (63). Crystal structure analysis revealed that a tetramer of Mn-SOD forms a ring of positive electrostatic charge around the active site. The replacement of glutamine 143 (Gln143) with aspartic acid showed the important role of Gln143 in the hydrogen bond network that involves tyrosin 34 in maintaining the catalytic efficiency of enzyme (67).

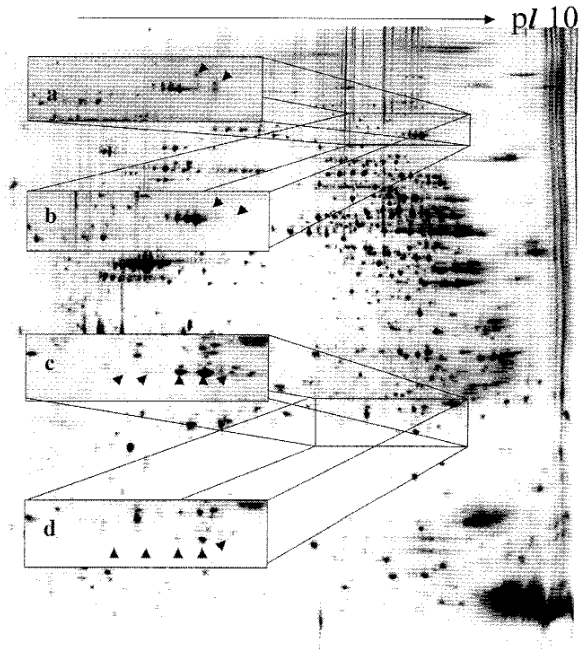
The SDO2 gene localised to 6q25.3-qter encodes Mn-SOD (68). N-terminus and C-terminus polymorphisms have been described to produce impairment of intracellular trafficking and inhibition of enzyme activity (69-71). Polymorphisms have been described representing a single nucleotide switch in the DNA region encoding a signal sequence with substitution of valine to alanine (Ala9Val) and threonine to isoleucine (Ile58Thr). They are implicated in the pathogenesis of Parkinson disease (72), cardiomyopathies (73), and breast cancer (74).

SOD2 gene expression is regulated by several transcription factors including NF-kB, AP-1, and Sp-1, and by DNA methylation of 5' CpG island on intron 2 (75-76) in response to stimuli of reactive oxygen species (ROS). Previous investigations were mainly focused on the protective role of Mn-SOD from cytotoxic ROS. Recent studies suggest that ROS and Mn-SOD may also have a role in cellular regulation and apoptosis. The latter may be driven in the mitochondria by several mechanisms, including disruption of electron transport, activation of caspase family proteases, and alteration of cellular redox status. Since the Mn-SOD is inducible by ROS, lipopolysaccaride, interleukin 1b (IL-1), tumour necrosis factor a (TNF-a), interferon gamma (IFN-y), and interleukin 6 (IL-6) (77), it thus suggests a putative protective role in cellular damage induced by drugs and radiation (78-79). Taken together these data suggest that the Mn-SOD overexpression may be correlated to radiotherapy and drug resistence of RCC, therefore Mn-SOD might be a target for immunotherapy.

Expression in human cancer

Several clinical disorders may show altered Ievels and activity of Mn-SOD, including diabetes, rheumatoid arthritis, epilepsy, osteoporosis, and cancer. Overexpression of Mn-SOD was described to decrease the malignant phenotypes of several cancers. St. Clair's group cloned and sequenced human Mn-SOD to study its regulation under oxidative stress in normaI and tumour cells. Using celI lines and transgenic mice expressing the human Mn-SOD gene, this group demonstrated that protection of mitochondria leads to a reduction of neoplastic transformation. This led to the concept of Mn-SOD as a tumour suppressor gene (80). Reverse transcription PCR in esophageal carcinoma showed reduced levels of Mn-SOD in conjunction with invasiveness (81). However, the potential tumour suppressor function of Mn-SOD is not clear since it is negatively regulated by another tumour suppressor gene product, the p53 protein (82). In contrast, high Ievels of this protein were found to correlate with poor prognosis in patients affected by glioblastoma (83), cervical carcinoma (84), metastatic gastric cancer (85), and central nervous system tumours (86). The Mn-SOD levels of expression were investigated in sera of RCC patients and did not show significant increase when compared to healthy volunteers (Sarto C., personal communication) (1). Studies of

Figure 2. The figure shows gel area zoomed with two multimeric (a) and five monomeric (c) isoforms of Mn-SOD separated from RCC. In the gel of normal kidney tissue only two monomeric (d) forms are visible, and multimeric forms (b) are not detected.



Mn-SOD Ievels in RCC were performed using immunogold stain by Oberley *et al.* (87). They observed variability of Mn-SOD Ievels in association with heterogeneity of celi types and mitochondria. This is in disagreement with recent data showing overexpression of Mn-SOD independently from the cell types (56). The diversity of Mn-SOD levels in various human cancers is most likely due to the combination of heterogeneity of genomic events and environmental changes. It modulates the activity of promoters and inhibitors of this protein differently in several tissues by different pathways.

Post-translational modifications

There are few studies describing post-translational modifications of Mn-SOD. This protein was separated and identified from various human cells and tissues by a combination of 2-DE and N-terminal sequence and MALDI-MS analysis or immunoblot analysis (77, 88-92). Different Mn-SOD isoforms with a variabihty of experimental p*l* and *Mr* were found in various studies. This could be due to either poor reproducibility of 2-D gels and eliectrophoresis or to post-translational modifications. The cysteine residues have reactive sulphydryls that can react with gel components giving rise to artefactual isoforms (66). A significant post-translational modification of tyrosine 34 residue by peroxynitrite nitration or oxidation was demonstrated to inhibit activity of Mn-SOD. This illustrates how investigation of either activity or protein levels is not sufficient to completely unravel biological processes . Analysis of RCC 2-DE maps pointed out both overexpression of Mn-SOD and additionai monomeric isoforms not detectable in normal kidney tissue (Fig. 2). Preliminary investigations by MALDI-TOF-MS and ESI-MS/MS of these isoforms after tryptic digestion did not reveal any post-translational modifications. However, trypsin generates a large peptide of 37 residues (157-194) absent in all the spectra. The investigation of this peptide and potential post-translational modifications is currently underway using pepsin and chymotrypsin prior to ES1-MS/MS.

Concluding remarks

Despite major advances in RCC molecular genetics, the discovery of an early specific plasmatic biomarker for the diagnosis and prognosis of RCC has so far been largely negative. This is most likely due to an enormous heterogeneity in the type of cells, and proteomic approaches should be now envisaged to further speed up the process of the search of this “Holy Grail”.

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