Genetic and epigenetic changes of GPX1 and GPX3 in human clear-cell renal cell carcinoma

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Aim. To find putative diagnostic and prognostic markers of cancerogenesis. Methods. Analysis of microarray and SAGE data, quantitative PCR (Q-PCR), bisulfite sequencing, methylation-specific PCR. Results. Bioinformatic analysis of microarray and SAGE database revealed that genes, encoding the glutathione peroxidase 1 and 3 (GPX1 and GPX3) were expressed at low levels in renal cancers. The relative gene expression of GPX1 and GPX3 that was widely inactivated in clear-cell renal cell carcinoma (ccRCC) was confirmed by Q-PCR. No correlation between expression levels and promoter methylation was found. It was found, however, that an allele with five ALA repeats in the N-terminal region of GPX1 is the most frequent polymorphic variant in ccRCC patients. Conclusions. Our data support the hypothesis that GPX1 and GPX3 are involved in tumorigenesis of ccRCC and could be putative TSGs (tumor suppressor genes) in renal cancer.

Keywords: renal cell carcinoma, genetic and epigenetic regulation, chromosome 3, quantitative real time PCR, methylation status.

Introduction. Renal cancer is among the ten most common causes of cancer-related death in adults [1]. The common subtypes of renal tumors are clear-cell renal cell carcinoma (ccRCC) (83.2%), papillary (11.3%) and chromophobe carcinoma (4.3%) [2]. The ccRCC is characterized by a variable and unpredictable clinical course. Patients with this disease have a poor 5-year survival [3]. Thus, accurate prediction of the course of disease is important for treatment. In spite of the large number of studies, the molecular mechanisms associated with tumor growth, metastasis, and progression of ccRCC are not sufficiently explored. One of the most important tasks is the identification of tumor suppressor genes (TSGs) [4].

One of the approaches to find a putative TSG is to analyze an open bioinformatic databases, for example, oligonucleotide microarrays (microchips) database and serial analysis of gene expression (SAGE). DNA-microarray technology, based on cDNA or oligonucleotide, allows obtaining quantitative information about the expression profiles in the studied samples. On the other hand, though the microarray technology allows an analysis of expression level of thousands genes, these genes usually have been already identified. SAGE is a technology for quantitative characterization of transcriptom, i.e. the totality of mRNA in a particular type of cell or tissue [5, 6].

We have used different approach to perform a large-scale search for changes of gene expression in renal cancer. This may lead to identification of the genes that could be involved in renal carcinogenesis and could serve as diagnostic and prognostic tumor markers. After the search in SAGE and Microarray databases, we confirmed our findings by Q-PCR. We have also investiga-
ted genetic and/or epigenetic changes of candidate genes in the experimental system.

Using the proposed approach, we analyzed 83 tumor samples in comparison to 84 normal tissues in 11 independent experiments.

**Materials and methods. Analysis of microarray and SAGE databases.** To analyze the databases «R Project» for Statistical Computing (www.r-project.org) was used. To process the initial microarray data available publicly the Bioconductor package was used. An additional normalization of processed data was done, using a method suited for Q-PCR. Differentially expressed genes were analyzed by limma package. The Limma gives a possibility to analyze many RNA targets simultaneously [7].

The following main steps were performed: resetting bioinformatics analysis in the context of general linear models with arbitrary units and contrasts of interest, deriving consistent, closed form estimators for the hyper parameters, using the marginal distributions of the observed statistics, reformulating the posterior odds statistic in terms of a moderated t-statistic in which posterior residual standard deviations are used in place of ordinary standard deviations.

SAGE map is a serial analysis of gene expression data repository supporting public use and dissemination of SAGE data. All of the SAGE libraries are present on the following website: ncbi.nlm.nih.gov/projects/SAGE/. The main idea was to compare all available libraries of normal kidney tissues with renal cancer libraries, using SAGE Digital Gene Expression Displayer (DGED) to find genes with different expression in kidney cancers.

**Tissue samples.** Surgically removed tumors and surrounding normal tissues were obtained from Kyiv National Urological Center (Kyiv, Ukraine). All tumor specimens were characterized according to the International System of Clinico-Morphological Classification of Tumors (TNM), based on the tumor-node-metastasis and staging classification of 1989 [8] and WHO criteria classification of 1999 [9]. The gene expression pattern was analyzed in 12 ccRCC samples and 12 corresponding normal tissues from the same patients: 6 samples were of stage 1–2 and 6 were of stage 3–4. The mean age of patients at diagnosis was 57.4 ± 11.6 (in the range of 36–69 years). «Normal» matched controls were obtained at the distance of minimum 2 cm from the tumor site and confirmed histologically as normal renal epithelial cells. The 13 ccRCC samples were investigated for methylation status and trinucleotide polymorphism (7 samples were of stage 1–2 and 6 samples were of stage 3–4). Three of these samples were studied, using bisulfite sequencing.

The present study was performed in accordance with the permission from the Ethical Committee of IMBG.

**Isolation of genomic DNA and total RNA.** Genomic DNA was isolated, using DNA purification Kit («Fermentas», Lithuania), according to the manufacturer’s recommendations. Total RNA was isolated from all fresh-frozen renal tumors and from normal tissues surrounding the tumors by homogenization with RNeasy Mini Kit («QIAGEN»), USA), according to the producer’s protocol. Quality of genomic DNA and total RNA was assessed by agarose gel electrophoresis; their concentration was measured, using a spectrophotometer Nano Drop ND-1000 («NanoDrop Technologies Inc.», USA). The samples, used for the Q-PCR reactions, were of high molecular weight and pure from contaminations (an OD 260/280 ranging from 1.6 to 1.8). From each RNA sample, 1 μg of total RNA was treated with DNAseI and reversely transcribed in duplicates, as well as negative controls without enzyme, using the RevertAid™ H Minus First Strand cDNA Synthesis Kit («Thermo Scientific», SE, Sweden).

**Analysis of gene expression levels.** We have used Q-PCR to study a relative gene expression, using «Bio-Rad» iQ5 (USA). The reactions were performed, using SYBR Green. TBP was used as a reference gene [11]. Primers for GPX1 and GPX3 were designed, using the Primer3 (http://frodo.wi.mit.edu/primer3/) and Oligo6.24 program and were synthesized by «Invitrogen» (BV, Netherlands). For mRNA detection the following primers were used:

- **GPX1 for:** 5’-CCAAGCTCATCACCTGGTCT-3’;
- **GPX1 rev:** 5’-TCGATGTCATGGTGCTTGGA-3’;
- **GPX3 for:** 5’-TACGAGTGCTGAGCCCTCAC-3’;
- **GPX3 rev:** 5’-CCAGAATGACCAGACCGAAT-3’;
- **TBP for:** 5’-GAACCACGGCACTGATTTTC -3’;
- **TBP rev:** 5’-CACAGCTCCACCATATTC-3’.

Primers for mRNA detection were selected to span for at least one big intron (more than 1 kb). Each Q-PCR reaction mix contained 12.5 μl of 2 × YBR Green PCR Master Mix («Fermentas»), forward and reverse primers.
at optimized concentrations of 400 nM, 10 ng/μl cDNA template and a sterile water to make a final volume of 25 μl. The reaction profile was: an initial step at 72 °C for 2 min, denaturation at 95 °C for 10 min, then 35 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s and extension at 72 °C for 40 s. To generate standard curves for the selected primers and the reference primers a log10 dilution series of cDNA was prepared at concentrations ranging from 1 ng to 100 ng. A GPXs expression level was estimated by the 2-ΔΔCT method of relative quantification [12].

Analysis of methylation status. Methyl-specific PCR for GPXs fragment amplification was carried out with primers specific to the methylated and unmethylated DNA sequences of GPXs CpG island. Blood gDNA treated with SssI-methyltransferase («New England Biolabs», UK) was used as a positive control. The methylation status of genes was determined in three selected samples by bisulfite sequencing as described earlier [10]. Bisulfite treatment was performed, using the EZ DNA Methylation Kit («Zymo Research Corporation», USA). PCR was carried out for 35 cycles, comprising 30 s denaturation at 94 °C, 30 s annealing at 56 °C, and 1 min extension at 72 °C. The cycling started by 2 min denaturation at 94 °C. The PCR products were purified, using the DNA Clean and Concentrator Kit («Zymo Research Corporation»), according to the manufacturer’s protocol. PCR products were cloned, using the TOPO TA Cloning Kit for Sequencing («Invitrogen»). Plasmid DNA was isolated, using a Zippy Plasmid Miniprep Kit («Zymo Research Corporation»). Sequencing was performed, using the BigDye Terminator Cycle Sequencing Ready Reaction kit v1.1 and ABI Prism 3100 Genetic Analyzer («Applied Biosystems», USA). The following primers were used for bisulfite sequencing of GPXs («Invitrogen»):

- **GPX3 BS for**: 5'-GGGATTTGATTTTATTATT TTGTTTAGATTTTGGTT-3';
- **GPX3 BS rev**: 5'-AAAAAAACCTTCTTCTCCC TTAATCATTCTA-3';
- **GPX1 BS rev**: 5'-CAAAAAACCTTACTCACA AAACCTCCTC-3';
- **GPX1 BS for**: 5'-AGTTTTTGGAAAGGTATTT TGGAT-3'.

Loss of heterozygosity. PCR was carried out for 28 cycles, comprising 30 s denaturation at 94 °C, 30 s annealing at 68 °C for GPX1 and 63 °C for GPX3, and 1 min extension at 72 °C. The cycling started by 2 min denaturation at 94 °C. One of the primers was labeled by Cy5 («IBA», USA):

- **GPX1 for**: 5'-Cy5GAAAAACTGCCCTTGCCACGT G ACC-3';
- **GPX1 rev**: 5'-CGAGAAGGCATAACCCGACTGG GC-3';
- **GPX3 for**: 5'-Cy5GATGTGAAGGCCACCTTGCTC-3';
- **GPX3 rev**: 5'-ATG GTG CTG GCC TGT CTA T-3'.

Denaturing PAAG electrophoresis was used to determine the exact size of trinucleotide repeats. The electrophoresis was conducted on automatic laser sequencing machine «ALF express» («Pharmacia Biotech», USA). The mixture, containing 3 μl of amplification product and 2 ml of loading buffer (0.01 % bromphenol blue, 0.01 % xylene cyanol, 98 % formamide), was denatured at 95 °C for 5 min before electrophoresis.

Statistical analysis. The nonparametric Wilcoxon test was used to compare mRNA expression of target and reference genes for the same sample. Then groups of samples were compared in respect to the average level of mRNA decrease (LDav) and the frequency of decrease (FD). The LD was calculated as 1/R, where R is an mRNA copy number ratio (R) of a target gene versus reference gene and reflects the n-fold factor by which the mRNA content decreased in the tumor compared to normal tissue [10]. The nonparametric Kruskal – Wallis and Mann – Whitney rank-sum tests were used to test mRNA differences (both LDav and FD) for each target gene, in tumors with and without metastases. The nonparametric Spearman’s criterion was used to calculate the coefficient of correlation between the LDav for each set of pairs of target genes. P-values < 0.05 were considered statistically significant. All statistical procedures were performed, using the BioStat software.

Results and discussion. Microarray and SAGE data analysis. To identify putative markers of ccRCC as targets for novel therapeutic drugs, we have investigated genome-wide expression profiles of all human genes in ccRCCs, using a bioinformatics analysis of cDNA microarray data. The results of 11 independent cases of analysis (such as GSE11985, GSE12114, GSE781, GSE12630 and others) were compared with the data reported for 84 normal and 83 tumor samples. In order to find genes with different expression level during kid-
ney carcinogenesis, we have also compared the kidney tumor library with 2 libraries of normal tissues, using the SAGE Digital Gene Expression Displayer (DGED). Bioinformatic analysis showed that the TBP gene may be used as a reference gene to investigate the gene expression pattern in human renal cell carcinomas, verifying the results that were published earlier [11].

The 12 genes, that followed a pattern reported in Microarray and SAGE databases, were found. 2 genes – GPX1 and GPX3 were chosen to study genetic and epigenetic changes, due to their function in progression and metastasing of renal tumor. Our data supported the hypothesis that these 2 genes are involved in tumorigenesis of ccRCC, due to the fact, that GPX1 and GPX3 expression was simultaneously decreased in renal cell carcinomas, according to microarray and SAGE analysis.

Levels of relative gene expression. The GPX1 expression decreased significantly (from 2 to 94 fold) at the mRNA level (LD) in 87 % of RCC samples (8 of 12, \( P < 0.002 \)) in comparison with surrounding normal tissue (Fig. 1), according to Q-PCR data. While no significant association was found between gene expression level and gender or age, it should be pointed out that GPX1 expression has a trend to correlate with the histological grade.

The GPX3 expression was down-regulated in all of 12 ccRCC samples in comparison with surrounding normal tissue (Fig. 2). Also no significant association was found between gene expression level and gender or age.

The obtained data on relative expression of GPX1 and GPX3 confirm the results of microarray and SAGE analysis.

To reveal possible mechanisms of gene expression decline, the methylation status of gene promoters was investigated, because it might be an epigenetic inactivation of these genes. It might be and/or a loss of heterozygosity as well.

Analysis of methylation status. To understand the mechanisms, underlying the observed down-regulation of GPXs in renal cancer samples, an analysis of promoter methylation was performed. CpG islands were identified in the exon1/intron1 region of GPX3. However, no CpG islands were identified in the promoter region up to 3,000 bp of GPX3. Methylation-specific PCR for GPX1 and GPX3 was performed for 13 ccRCC samples and surrounding normal tissues. No methylation was found in any of renal cancer samples. Bisulfite sequencing was performed on 3 samples (2, 9, and 11) from renal cancer and 3 normal tissues. The results of methylation-specific PCR confirmed the absence of changes in methylation profile, indicating that other mechanism of inhibition of gene expression should be responsible, for example, genetic changes.

Loss of heterozygosity study. The loss of heterozygosity (LOH) method was used for a search for deletion. However, no LOH was found for the GPX1 and GPX3 genes.

Trinucleotide repeats determining. The allele’s analysis was performed to investigate the trinucleotide polymorphic locus in GPX1 gene in the 13 patients with ccRCC. The cancer-associated allele with five Ala repeats was found in 61.5 % samples of renal cancer. Alleles 6 and 7 constituted a proportion of 27 and 11.5 % respectively. The results are presented in Fig. 3 and Table.
Glutathione peroxidase functions in the detoxification of hydrogen peroxide, and it is one of the most important antioxidant enzymes in humans. Consequently, glutathione peroxidases might have dual role in the regulation of hydroperoxide levels. GPXs are down-regulated in different tumors, such as breast, gastric, and colorectal cancers [13, 14], prostate cancer [15], thyroid cancer of different origin [16, 17], and endometrial carcinoma [18]. Since \textit{GPX3} was always highly expressed in the corresponding healthy tissues, it has been suggested to exhibit tumor suppressor activity. Its main role would be a prevention of cancer initiation, caused by ROS-mediated DNA damage. Glutathione peroxidases play a critical role in detoxifying reactive oxidative species and maintaining the genetic integrity of mammalian cells. Cancer cells produce high amounts of reactive oxygen species (ROS) and evade apoptosis. Hydroperoxides support proliferation, invasion, migration and angiogenesis, but at higher levels they can induce apoptosis, thus being pro- and anti-carcinogenic at the same time. Metastasizing and also apoptosis are inhibited by all GPXs. GPXs that mediated through regulation of other protein activities, may be important for early stages of inflammation-mediated carcinogenesis [19].

A transcription of \textit{GPX1} is also regulated by oxygen tension. Specifically, the human \textit{GPX1} gene has two oxygen response elements (OREs), which under normoxic conditions are important for transcription [20]. Further, it has been proposed that hypoxia-induced suppression of \textit{GPX1} transcription may contribute to reperfusion injury after low oxygen tension in cardiomyocytes [21]. Interestingly, hyperoxia enhances \textit{GPX1} transcription in human umbilical vein endothelial cells through a mechanism independent of the ORE [22], suggesting that \textit{GPX1} transcription may be regulated in response to oxygen tension by more than one mechanism.

Analysis of the novel \textit{GPX3} promoter identified the Sp-1- and hypoxia-inducible factor-1A (HIF1A) binding sites, as well as the redox-sensitive metal response element and antioxidant response element. HIF1A, activated by hypoxia, was identified as a strong transcriptional regulator of \textit{GPX3} expression, leading to almost 3-fold increase in the expression levels after 24 h of hypoxia compared with normoxic conditions [23]. It was reported that promoter methylation of GPXs family genes, namely \textit{GPX4} and \textit{GPX2}, was the main mechanism of expression loss in breast cancer [24]. In our research, the methylation of promoter region was not found. Therefore, the decline of expression, probably, could be conditioned by protein interactions. It is known that the oxygen response element sequences bind a nuclear complex that includes the nuclear factor, Ku [25]. So, GPXs expression is very sensible to the amount of oxygen in a cell. As the innidiation and hypoxic effects in the cell are closely bound, the expression level of \textit{GPX1} and \textit{GPX3} allows a judgement about a possible innidiation. As was shown before, \textit{GPX1} gene is characterized by polyalanine sequence polymorphism in the N-terminal region, which includes three alleles with five, six or seven alanine (Ala) repeats. As was reported in [26], this locus is polymorphic and the allele with five Ala repeats is associated with breast cancer risk. The results of our research confirmed predominance of cancer-associated allele in ccRCC samples.
Conclusions. The results of our study confirm the data of microarray and SAGE analysis for the GPX1 and GPX3 genes. In the present work we have shown the essential decrease in the relative expression level of GPX1 and GPX3 in ccRCC.

Establishment of possible genetic and epigenetic mechanisms that contribute to the expression decline has shown that promoter methylation was not a key mechanism. It was shown that the rarest allele trinucleotide polymorphism was the allele with 7 Ala repeats for both genes. In the present work we have shown the expression data of microarray and SAGE analysis for the GPX1 gene, and it has shown that promoter methylation was not a key mechanism that contribute to the expression decline of this gene in ccRCC. It was shown that the rarest allele trinucleotide polymorphism was the allele with 7 Ala repeats for both genes.

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