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# GENETIC AND EPIGENETIC CHANGES OF *NKIRAS1* GENE IN HUMAN RENAL CELL CARCINOMAS

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**Renal cell carcinoma (RCC) is the most common malignant tumor of kidney associated with the worst clinical outcome. No molecular markers for RCC diagnostics and prognosis that could be applied in clinics were described yet. Large-scale screening of 3p human chromosome genes/loci in RCC and histologically normal tissues surrounding the tumors using NotI-microarray approach demonstrated that *NKIRAS1* gene contained the largest percent of genetic/epigenetic changes in RCC tumor cells. *Aim:* To validate the results of NotI microarray analysis and study genetic, epigenetic changes, and the expression level of *NKIRAS1* gene in human RCC samples. *Methods:* DNA and RNA were isolated from freshly-frozen renal tumors' samples (n = 12) and from normal tissues surrounding the tumors. Epigenetic changes (methylation status) of *NKIRAS1* were detected by bisulfite sequencing. Genetic changes and expression level were analyzed by Quantitative real-time PCR (qPCR) with SYBR Green. For relative quantification $2^{-\Delta\Delta CP}$ method was used. Nonparametric tests (Wilcoxon, Kruskal — Wallis and Mann — Whitney) were applied for statistical data analysis using the BioStat software. *Results: NKIRAS1* expression was downregulated in 75% of RCC samples (9 of 12) compared with surrounding normal tissue. High grade tumors (3 and 4) showed lower expression of *NKIRAS1* at the mRNA level than tumors of low grade (1 and 2). No significant association was found between gene expression level and gender or age. Analysis of *NKIRAS1* gene copy number was performed in 19 tumor samples. Changes in the copy number of *NKIRAS1* gene were observed in 64% (9 of 14) of cRCC samples. 9 samples displayed ratio ($< 0.85$ and $\geqslant 0.35$), thus were considered as hemizygous deletions. 3 samples showed ratio ($> 0.85$) and were considered as normal copy number. Changes in *NKIRAS1* gene copy number were detected in all 3 benign oncocytomas, 1 papillary cancer and 1 sarcoma, where hemizygous deletion was observed. No changes in methylation status of *NKIRAS1* were found in RCC. *Conclusions:* We have validated the results of NotI microarray analysis of *NKIRAS1* gene in RCC. It was shown the decreased expression level of *NKIRAS1* in this type of tumor.**

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

Renal cell carcinoma (RCC) is the most common malignant kidney tumor in adults. It stands for approximately 3% of all human malignancies. Among cancers of the urinary system, RCC is associated with the worst clinical outcome [1]. The incidence of RCC is increasing and it is estimated that RCC accounts worldwide for 95,000 cancer-related deaths per year [2].

At present, surgical resection is the most effective treatment for localized RCC tumors, but no satisfactory treatment is available for patients with advanced-stage tumor. Some therapies for RCCs have achieved a response rate of 20%, but severe adverse reactions are frequent and prognosis for patients does not seem to have improved overall. Although tumor stage is considered to be the most informative prognostic factor, little is known about the underlying molecular mechanisms of renal carcinogenesis.

The most common histological subtypes of RCC include clear cell (80%), papillary (> 10%), and chromophobe (< 5%) carcinomas. These histological subtypes are genetically and biologically different [3, 4]. Many genes and signaling pathways are known to be involved in RCC initiation and progression [5]. However, until today no tumor suppressor gene, which malfunction could be responsible or at least contributing to the development of clear cell renal cell carcinoma (ccRCC) has been identified. Several potential candidates were proposed, such as *VHL* (von Hippel Lindau), *FHIT* (fragile histidine triad), *TTRC1* (two-three-renal-cancer-1), *DUTT1* (deleted in U-twenty twenty), locus *NCR-1* (non papillary renal cell carcinoma 1), and *RASSF1A* (RAS association family 1) [6–9].

Papillary RCCs are characterized by trisomies (chromosomes 3q, 7, 12, 16, 17, and 20) and loss of the Y chromosome [10]. Chromophobe RCCs are characterized by monosomy of multiple chromosomes (1, 2, 6, 10, 13, 17, and 21). Clear-cell RCCs are characterized by loss of genetic material on the chromosomes 3, 8, 9, 10. Fifty percent of clear-cell RCCs show somatic mutations of *VHL* gene, and, moreover, the 10% to 20% of these tumors show inactivation of the *VHL* gene [11]. Loss of heterozygosity on chromosomes 8p or 9p provides prognostic significance in patients with locally advanced ccRCC and *PTEN/MMAC1* (chromosome 10) inactivation that may play a role in the ccRCC progression [12]. However, these molecular markers have not yet gained general use in RCC diagnostics and prognosis.

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*Abbreviations used:* ccRCC – clear cell renal carcinoma; NKIRAS1 – NF-κB inhibitor interacting Ras-like 1; RCC – renal cell carcinoma; TBP – tata-box binding protein; *VHL* – von Hippel Lindau gene.

The most predominant type of RCC, clear cell carcinoma, was chosen for this study and the large-scale analysis of gene expression profiles was performed. Similar studies, reported by other groups earlier, have already identified some genes that might be useful for prognostic purposes or for classification of RCCs [13–15]. Consequently, relatively little is known about molecular expression profiles associated with tumor growth and metastatic progression of ccRCC.

The largest percent of genetic aberrations associated with ccRCC includes 3p loss or changes. DNA methylation/deletion is a key mechanism to inhibit the expression of tumor suppressor genes in cancer; hence DNA methylation/deletion markers have been applied in cancer risk assessment, early detection, prognosis, and prediction of response to cancer therapy.

Genetic/epigenetic changes could be investigated, using microarrays analysis. NotI microarrays that were recently developed by us, give the new possibilities for large scale study of deletion/methylation patterns in normal compared to pathological cells [16].

Using the NotI microarrays, we have found that a locus associated with the NF-κB inhibitor interacting Ras-like 1 gene (*NKIRAS1*) had the largest percent of genetic/epigenetic changes, and exclusively in RCC [17–19]. The *NKIRAS1* gene encodes a Ras-like protein that acts as a potent regulator of NF-kappa-B activity. [20–22]. Function of *NKIRAS1* in carcinogenesis is unknown and its characterization can shed some light on the mechanism of epithelial cancer development.

In the present work we further analyzed the expression level, deletion and methylation status of the putative tumor suppressor gene *NKIRAS1* in kidney cancers.

## MATERIALS AND METHODS

***Tissue samples.*** Frozen surgically removed tumors and surrounding normal tissues were obtained from Kyiv National Urological Center (Kyiv, Ukraine). Tumors were histologically classified according to the WHO criteria by TNM classification. This series included 3 oncocytomas (benign), 18 conventional clear cell tumors (2 with sarcomatosis), 1 papillary tumor, and 1 sarcoma. The mean age of patients at diagnosis was 57.4 ± 11.6 (range 36–69 years), with a female/male ratio of 1.3. The present study was performed in accordance with the institutional Ethical Committee permission. All the samples enrolled in this study were processed anonymously.

***Isolation of DNA and RNA.*** DNA was isolated using phenol-chloroform extraction. Total RNA was isolated from all fresh-frozen renal tumors and from normal tissues surrounding the tumors by homogenization with an acid guanidinium thiocyanate-phenol-chloroform mixture as described [23]. Samples used for the qPCR reactions were of high molecular weight (unsheared band of undigested DNA visible on a 0.5% agarose gel) 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 reverse transcribed in duplicates, as well as negative controls without enzyme, using the reverse transcriptase kit (Fermentas).

***Methylation status***. The methylation status of *NKIRAS1* was determined in three selected samples by bisulfite sequencing as described earlier [24, 25]. Bisulfite treatment was performed using an 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 a DNA Clean and Concentrator Kit (Zymo Research Corporation) according to the manufacturer's protocol, and the PCR products were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen BV, Netherlands). Plasmid DNA was isolated using a Zyppy Plasmid Miniprep Kit (Zymo Research Corporation, USA). Sequencing was performed using a BigDye Terminator Cycle SequencingReady Reaction kit v1.1 and ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA).

***Primer design.*** Primers for gene *NKIRAS1* were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and PrimerQuest (http://eu.idtdna.com/Scitools/Applications/ Primerquest/), and Oligo6.24 program. For genomic sequence following primers were used: forward 5'-cgagaggtgagagagttggc-3', reverse 5'-tgcgtgaaacaaacctgttct-3'. For mRNA detection following primers were used: forward 5'-ctttcaaagagtggagcttctg-3', reverse 5'-tttccgatctgtaacagtcacc-3'. Primers for mRNA detection were selected to span at least one big intron (more than 1kb).

***Expression level analysis.*** Reactions were performed using SYBR Green. *TBP* was used as a reference gene [26]. Each qPCR reaction comprised 12.5 μl 2x SYBR Green PCR Master Mix (Fermentas), forward and reverse primer at optimized concentrations of 400 nM, 10 ng/μl cDNA template and sterile water up to a final volume of 25 μl. The qPCR reactions were performed using Bio-Rad iQ5. The reaction profile was: initial step, 72 °C for 2 min, denaturation 95 °C for 10 min, then 35 cycles of denaturing 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. Reference gene *TBP* was previously found to be stably expressed in renal tumors. Each cDNA sample was analyzed in triplicate by real-time PCR, and detected with SYBR Green Master Mix. Each qPCR experiment contained duplicates of the notemplate-controls and patient samples. Quantification was based on the increased fluorescence, which was measured and recorded using the Bio-Rad iQ5.

***Copy number analysis.*** Quantitative real-time PCR (qPCR) was used for the quantification of marker copy numbers and has been shown to be precise enough to discriminate between two and one allele copies. The parameter CP (threshold cycle) is defined as the cycle number required for dye fluorescence to become higher than background fluorescence level.

Reactions were performed using SYBR Green and detected with SYBR Green Master Mix (Fermentas) with similar reaction conditions as for expression analysis. The method is based on the inverse exponential relationship that exists between initial quantity (copy number) of target sequence copies in the reactions and corresponding CP determinations — the higher the starting copy number of DNA target the lesser the CP value. This method was used to determine target sequence copy number in tumor DNA sample relative to the normal DNA from the same patient (calibrator) and relative to an endogenous control sequence (reference) — TBP. Quantification was based on the increased fluorescence, which was measured and recorded using the Bio-Rad iQ5. According to histology analysis contamination of tumour samples with normal stroma and lymphocytes can reach up to 30–40%. Therefore, alleles were taken as homozygously deleted if the highest value of calculated range was below 0.5 and hemizygously deleted if this value was below 1.0. An allele was considered as amplified/multiplied if the lowest value of the range was over 1.0 [27].

***Data analysis.*** Copy numbers in *NKIRAS1* gene and its expression level was estimated by the $2^{-\Delta\Delta CP}$ method of relative quantification [28]. The relative expression ratio (*R*) of a target gene is calculated based on *E* and the CP deviation of an unknown sample versus a control, and expressed in comparison to a reference gene. For the ΔΔCP calculation to be valid, two important parameters must be considered beforehand. First, the efficiency of a given PCR amplification must be close to 100%, and second, the relative efficiency must be optimal. That is the amplification efficiencies of the target and reference genes must be approximately equal. PCR efficiencies (E) were calculated from the given slopes, according to the equation:

$$E = (10^{(-1/slope)} - 1) \times 100,$$

where E = 100 corresponds to 100% efficiency. Plots of the log DNA and cDNA dilutions *vs* ΔCP were made for each primer pair. Also for every reaction efficiency was calculated using sigmoidal and exponential models from qPCR package [29]. Further calculations were performed using Microsoft Excel. The range given for the probes was determined as $E^{-\Delta\Delta CP}$ with ΔΔCP + S and ΔΔCP-S, where s is the standard deviation of the ΔΔCP value.

***Statistical analysis.*** 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 average level of mRNA decrease (LDav) and the frequency of decrease (FD). The LD was calculated as 1/R and reflects the n-fold factor by which the mRNA content decreased in the tumor compared to normal tissue. Nonparametric Kruskal — Wallis and Mann — Whitney rank-sum tests were used to test mRNA differences (both LDav and FD) for each target gene and with and without metastases. Nonparametric Spearmen's criterion was used to calculate the coefficient of correlation between the levels of mRNA decrease (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 [30].

## RESULTS AND DISCUSSION

NotI microarray approach allows the search for hemizigous deletion/methylation that can indicate a reduction or loss of expression of the certain genes. Previously, we have found changes in 137 of 181 NotI clones from human chromosomes 3 [17]. Heterozygous deletion/methylation events were observed most frequently (428 cases); amplifications (24 cases) and homozygous deletion/methylation events (24 cases) were observed rarely. NotI microarray data analysis produced a profile of the changes in chromosome 3 genes/loci in the epithelial cancer samples. Among them, 27 genes/loci showed changes in more than 30% of samples.

To validate NotI microarray results we have chosen the NF-κB inhibitor interacting Ras-like 1 (*NKIRAS1*) gene that showed the highest percentage of genetic/epigenetic changes. Gene *NKIRAS1* is localized in 3p24.2 locus on the human chromosome 3, which has the greatest amount of changes in epithelial cancers. Thus, 78% of cases demonstrated hemizigous deletion/methylation in the NotI locus associated with *NKIRAS1* gene [17].

We selected several samples with genetic/epigenetic changes for bisulphite sequencing and found an absence of DNA methylation in all samples, both in tumor and normal tissue (data not shown). To explain the high percent of changes obtained by NotI microarray approach we measured the expression level and estimated the copy number of *NKIRAS1* gene.

Significant (from 2 to 94 fold) decrease of the mRNA level (LD) of gene *NKIRAS1* was observed in 75% of RCC samples (9 of 12, $P < 0.002$) in comparison to surrounding normal tissue (Table 1). *TBP* expression level was found to be constant in tumor and normal renal tissue. While no significant association was found between gene expression level and gender or age, it should be pointed out that *NKIRAS1* expression correlated with histological grade. Thus, tumors of grade 3–4 had lower *NKIRAS1* mRNA level than tumors of grade 1–2 (Figure).

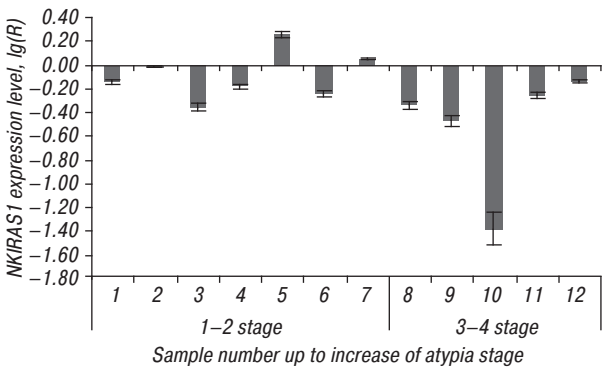


**Figure.** *NKIRAS1* expression (n = 12) in renal cell carcinoma

Analysis of *NKIRAS1* copy number was performed in 19 samples (3 benign oncocytomas, 1 papillary cancer, 1 sarcoma, and 14 ccRCCs), using qPCR (Table 2). *NKIRAS1* copy number decrease was observed in

64% (9 of 14) of cRCC samples: 9 samples displayed ratio < 0.85 and ⩾ 0.35 and were considered as hemizigous deletions. 3 samples had ratio > 0.85 and were considered to have unmodified copy number status. Copy number changes were found in all 3 benign oncocytomas, 1 papillary cancer, and 1 sarcoma, where hemizigous deletions were observed. All these data suggest that *NKIRAS1* gene could be involved in the carcinogenesis as a tumor suppressor gene.

The *NKIRAS1* gene encodes a Ras-like protein that acts as a potent regulator of NF-kappa-B activity. Functions of *NKIRAS1* in carcinogenesis are still unknown. Mutations of other members of the Ras gene family (*HRAS*, *KRAS2* or *NRAS*) are identified in approximately of 15% of human neoplasm cases [31]. Here we describe a novel member of this family which is associated with tumor growth and metastasis in epithelial cancer.

We have reported earlier that the CpG islands in the *ITGA9* gene/locus were homozygously methylated in one of the tissue samples, heterozygously methylated in the second sample, and unmethylated in the third sample from colorectal cancer [32]. These results correlated with the microarray results. The ratio of the hybridization signal intensity for tumor/normal tissues was 0.2, 0.55 and 0.97 for the three samples. No methylation of CpG islands was revealed for the corresponding normal tissues as well as no deletions were found. Our work demonstrated that the NotI microarray produces valuable data for analysis. The NotI microarray protocol might be useful as a primary screening method due to its ability to determine the global genetic/epigenetic changes in a genome. This may lead to the subsequent selection of gene candidates that may participate in carcinogenesis.

In summary, we have found that *NKIRAS1* is downregulated in malignant renal tumors and copy number of *NKIRAS1* gene is decreased in RCC. Due to the results of NotI microarray analysis *NKIRAS1* was selected for further investigation as a gene with the highest number of genetic alterations in epithelial tumors. We plan to analyze the association of *NKIRAS1* expression with the age of patients, and perform further studies to test if *NKIRAS1* gene expression could be regarded as diagnostic marker for RCC. The function of this protein in the development of renal cancer should be elucidated further.

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**Table 1.** *NKIRAS1* expression in renal cell carcinomas

| Sample number | TNM | Sex | Age | Pathohistology of renal cancer | Stage of atypia | R, Expression level |
|---|---|---|---|---|---|---|
| 1 | $T_3N_0M_0$ | f | 52 | Conventional clear cell cancer | 1 | 0.72 (0.62–0.81) |
| 2 | $T_2N_0M_0$ | f | 57 | Conventional clear cell cancer | 1 | 0.97 (0.87–1.08) |
| 3 | $T_2N_0M_0$ | m | 57 | Conventional clear cell cancer | 1 | 0.44 (0.35–0.52) |
| 4 | $T_2N_0M_0$ | f | 57 | Conventional clear cell cancer | 1 | 0.66 (0.50–0.81) |
| 5 | $T_2N_0M_0$ | f | 68 | Oncocytoma (benign) | 2 | 1.81 (1.52–2.09) |
| 6 | $T_3N_0M_0$ | f | 66 | Conventional clear cell cancer | 2 | 0.57 (0.47–0.67) |
| 7 | $T_2N_0M_0$ | f | 69 | Conventional clear cell cancer | 2 | 1.12 (0.76–1.47) |
| 8 | $T_3N_0M_0$ | f | 38 | Papillary cancer | 3 | 0.46 (0.35–0.57) |
| 9 | $T_2N_0M_0$ | m | 46 | Conventional clear cell cancer | 3 | 0.34 (0.24–0.43) |
| 10 | $T_3N_0M_0$ | m | 51 | Conventional clear cell cancer with sarcomatosis | (3–4) | 0.042 (0.041–0.043) |
| 11 | $T_3N_0M_0$ | f | 61 | Conventional clear cell cancer | 4 | 0.55 (0.44–0.67) |
| 12 | $T_2N_1M_1$ | m | 58 | Conventional clear cell cancer with adrenal metastasis | 4 | 0.74 (0.52–0.95) |

**Table 2.** Summary of NotI-microarray results and relative DNA calculation results for RCC samples

| Sample number | TNM | Sex | Age | Pathohistology of renal cancer | Stage of atypia | NotI microarray results | Real-time results |
|---|---|---|---|---|---|---|---|
| 1 | $T_3N_0M_0$ | f | 52 | Conventional clear cell cancer | 1 | 0.68 | 0.36 (0.30–0.43) |
| 2 | $T_2N_0M_0$ | m | 46 | Conventional clear cell cancer | 1 | 0.83 | 0.74 (0.68–0.82) |
| 3 | $T_2N_0M_0$ | f | 57 | Conventional clear cell cancer | 1 | 0.87 | 0.58 (0.56–0.61) |
| 4 | $T_2N_0M_0$ | m | 57 | Conventional clear cell cancer | 1 | 0.93 | 0.54 (0.53–0.55) |
| 5 | $T_2N_0M_0$ | f | 68 | Oncocytoma (benign) | 2 | 0.64 | 0.70 (0.63–0.77) |
| 6 | $T_2N_0M_0$ | f | 51 | Oncocytoma (benign) | 2 | 0.75 | 0.44 (0.28–0.60) |
| 7 | $T_2N_0M_0$ | m | 60 | Oncocytoma (benign) | 2 | 0.62 | 0.62 (0.53–0.70) |
| 8 | $T_2N_0M_0$ | f | 36 | Sarcoma | 2 | 0.49 | 0.45 (0.39–0.50) |
| 9 | $T_3N_0M_0$ | f | 66 | Conventional clear cell cancer | 2 | 0.62 | 0.44 (0.37–0.52) |
| 10 | $T_2N_0M_0$ | f | 60 | Conventional clear cell cancer | 2 | 0.79 | 0.57 (0.56–0.59) |
| 11 | $T_3N_0M_0$ | m | 66 | Conventional clear cell cancer | 2 | 0.64 | 0.35 (0.27–0.43) |
| 12 | $T_2N_0M_0$ | m | 55 | Conventional clear cell cancer | 2 | 0.84 | 1.09 (1.08–1.10) |
| 13 | $T_3N_0M_0$ | f | 38 | Papillary cancer | 3 | 0.88 | 0.16 (0.11–0.21) |
| 14 | $T_3N_0M_0$ | m | 65 | Conventional clear cell cancer | 3 | 0.77 | 1.01 (0.72–1.28) |
| 15 | $T_3N_0M_0$ | m | 56 | Conventional clear cell cancer | 3 | 0.94 | 0.79 (0.68–0.89) |
| 16 | $T_2N_0M_0$ | m | 46 | Conventional clear cell cancer | 3 | 0.78 | 0.34 (0.33–0.35) |
| 17 | $T_3N_0M_0$ | f | 60 | Conventional clear cell cancer | 3 | 0.79 | 0.34 (0.33–0.35) |
| 18 | $T_2N_0M_0$ | m | 60 | Conventional clear cell cancer with sarcomatosis | 4 | 0.76 | 0.41 (0.37–0.46) |
| 19 | $T_3N_0M_0$ | f | 61 | Conventional clear cell cancer | 4 | 0.72 | 0.89 (0.72–1.06) |

Note: Light grey color was used for samples, which displayed ratio < 0.35 and were considered as homozygous deletions. Dark grey color was used for samples, which displayed ratio < 0.85 and ⩾ 0.35 and were considered as hemizigous deletions. White color was used for samples, which had ratio > 0.85 and were considered to have unchanged copy number status.

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