Methylation pattern of the putative tumor-suppressor gene *LRRC3B* promoter in clear cell renal cell carcinomas

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Abstract. The leucine rich repeat containing 3B (*LRRC3B*) gene is a putative tumor suppressor located on human chromosome 3 in the 3p24 region. *LRRC3B* is frequently altered in colon and gastric cancers and also in leukaemias. In this study we investigated the promoter region methylation as a possible mechanism of *LRRC3B* gene inactivation in clear cell renal cell carcinomas. We found that the *LRRC3B* gene promoter was methylated in 43% of clear cell renal carcinoma samples. However, no correlation between DNA methylation and *LRRC3B* expression was found.

Introduction

Renal cell carcinoma (RCC) is among the ten most common type of cancer and accounts for 3% of all adult malignancies (1). The hallmark of RCC is a strong resistance to chemotherapy and radiotherapy. The pattern of RCCs includes non-papillary and papillary carcinomas. In both cases, malignant cells arise from the epithelium of the proximal section of the renal tube. The most common type among non-papillary RCCs is clear cell RCC, which accounts for 75-80% of RCCs. Other types of RCCs are papillary (10%) and chromophobe (4-5%) (2).

The main features of clear cell RCCs are loss of function of chromosome 3 and gain-of-function of chromosomes 5q and 7 (3). Human chromosome 3 contains several tumor-suppressor genes, including *VHL*, *RASSF1*, *DLEC1*, *FHIT*, *G21* and *RBSP3*, which are inactivated through genetic alterations or epigenetic changes in clear cell RCCs (4-10). The loss of the short arm of chromosome 3, due to deletions or unbalanced translocations, is a typical event in clear cell RCCs. The most frequently lost regions are 3p12-14, 3p21.3 and 3p25 (11).

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Previously, global scanning of genetic and epigenetic changes of human chromosome 3 has been performed by NotI-microarray technology in RCC (12). Among genes with a high score of alterations, the leucine rich repeat containing 3B (*LRRC3B*) gene was identified as one of the most frequently altered genes in RCCs (9). Our study aimed to investigate DNA methylation of the promoter region of the *LRRC3B* gene in clear cell RCCs. We demonstrated that the *LRRC3B* gene is hypermethylated in clear cell RCCs. However, no correlation between changes in expression and methylation status of *LRRC3B* was found in these tumors.

Materials and methods

Thirty-five surgically excised tissue samples of clear cell RCCs were used in the present study. All tumor samples were paired with non-malignant tissues, which were considered to be normal tissue samples. Samples of clear cell RCCs and adjacent non-malignant tissues were obtained from patients who had undergone kidney resection owing to renal cancer in the Institute of Urology (Kiev, Ukraine). Immediately following surgery, tissue samples were frozen in liquid nitrogen and stored at -70˚C. No chemotherapy or radiotherapy was conducted on any patients prior to surgery. Each sample was characterized histologically.

The genomic DNA isolation was performed as previously described (13). Briefly, 0.3-0.5 g of tissues was homogenized following freezing in liquid nitrogen and lysed in buffer containing 10 mM Tris-Cl (pH 8.0), 0.1 M ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS) for the duration of 20 min, followed by proteinase K (final concentration 100 μg/ml) (Fermentas, MD, USA) which was added to the lysates. The reaction mix was incubated for 3 h at 50˚C. DNA was extracted twice with phenol (pH, 8.0) and twice with chloroform. Precipitation of genomic DNA was performed by adding ammonium acetate up to 1.5 M and 3 volumes of ethanol. DNA was washed twice with 70% ethanol and dissolved in TE buffer.

The methylation status of the CpG-island of the *LRRC3B* gene was assessed by methyl-specific polymerase chain reaction (PCR), as previously described (14). To perform methyl-specific PCR (MSP) we designed primers for the

CpG-island of the promoter region of the *LRRC3B* gene: LRRC3B M-F 5'-GGTGCGAGGAAGGTAGGC-3'; LRRC3B M-R 5'-ACCAATACCTCGCCGACG-3'; LRRC3B U-F 5'-GG GTGTGAGGAAGGTAGGT-3'; and LRRC3B U-R 5'-CCAA CCAATACCTCACCAACA-3'.

The PCR mixture contained 1X PCR Dream buffer (Fermentas), 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 0.3 μM primers, 100 ng of modified DNA and 1 U of DreamTaq polymerase (Fermentas). Amplification was performed over 40 cycles (30 sec at 95˚C, 30 sec at 63˚C and 30 sec at 72˚C), initiated with DNA denaturation at 95˚C for 4 min. The final extension was at 72˚C for 5 min. Electrophoresis of the PCR samples was carried out in 12% polyacrylamide gels.

To verify methyl-specific PCR data the MSP sequencing assay was performed. Briefly, PCR products, obtained with primers for methylated DNA were cloned into pJET 1.2 vector. For each sample, at least 5 clones were selected and sequenced.

Genomic DNA was treated with sodium bisulfite (DNA Methylation kit, EZNA) and subjected to amplification with primers for the CpG-island with a product of 489 bp, containing two *Not*I sites: LRRC3B-BS For: 5'-GTTTTTGTTTGT TTTTTTTGTAAGGTTA-3'; LRRC3B-BS Rev: 5'-ACTAAT ATAATAATTCTCCTCTACTTATTCCTTAA-3'.

Amplification was performed under the following conditions: 95˚C for 4 min, 35 cycles; 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 60 sec. The final extension step involved 72˚C for 7 min. The reaction mixture contained 1X RedTaq buffer (Sigma, St. Louis, MO, USA), 0.2 μM of dNTPs, 0.8% dimethyl sulfoxide (DMSO), 1 U of RedTaq polymerase (Sigma) and 100 ng of bisulfite-treated DNA. The PCR products were purified from agarose gels with a DNA purification kit and cloned into the pGEM-T vector (Promega, Madison, WI, USA). At least 8 individual colonies were isolated and sequenced for each sample.

To assess expression of the *LRRC3B* gene in clear cell RCCs quantitative PCR (q-PCR) was used. Reverse transcription reactions were performed using 2 μg of total RNA by RevertAid H MinusReverse Transcriptase kit (Fermentas) according to manufacturer's instructions. Obtained complementary DNA (cDNA) was ethanol precipitated and diluted in 10 μl of mQ H_2O. Amplification was performed with the following primers: LRRC3B q-F 5'-CACACCCCTAAG CATACG-3'; and LRRC3B q-R 5'-TGTCTCTTCATTATTTCT TTCCTTG-3'.

The reaction of amplification contained 1X SYBR Green (Fermentas), 0.4 μM of each primer and 500 ng of cDNA. The conditions of q-PCR were 95˚C for 10 min, for 45 cycles; 95˚C for 15 sec, 59˚C for 20 sec and 72˚C for 30 sec. The quality of PCR products was checked by melting curve analysis. Q-PCR data were processed with R-package (2.12.1. version) as described by Spiess *et al* (15).

Results

Through the use of NotI-microarray technology we previously demonstrated that the *LRRC3B* gene locus was altered in 57% of RCC samples (9).

To study DNA methylation of the *LRRC3B* gene promoter region the MSP assay was used. A total of 35 samples of clear cell RCCs, with tumor stages T1N0M0 (3 tumors), T2N0M0 (20 tumors) and T3N0M0-T3NxMx (12 tumors), were assayed by MSP. It has been previously demonstrated that a critical promoter of the *LRRC3B* gene is between -195 and +323 bp (16), and contains two CpG-islands. Therefore, primers for methylated and unmethylated DNA of the *LRRC3B* gene promoter region (from -256 to -105 bp) were used in the MSP assay. Methylation of the *LRRC3B* gene was detected in 15 out of 35 clear cell RCC samples (43%). Results on *LRRC3B* methylation are presented in Table I and Fig. 1A.

To validate the results of the MSP assay a sequencing of methylated PCR product for 7 samples was performed. Sequencing confirmed the accuracy of the DNA methylation determination by MSP assay. The MSP product of the meth-

Table I. Methylation status of the *LRRC3B* gene in clear cell renal cell carcinomas.

No.	Stage	DNA methylation status
1	T3N0M0	-
2	T2N0M0	+
3	T3N0M0	+
4	T3N0M0	+
5	T2N0M0	+
6	T3N0M0	+
7	T2N0M0	+
8	T2N0M0	-
9	T3N0M0	-
10	T2N0M0	+
11	T3N0M0	+
12	T3N0M0	-
13	T3N0M0	-
14	T2N0M0	-
15	T1N0M0	-
16	T2N0M0	-
17	T1N0M0	-
18	T3N0M0	-
19	T2N0M0	-
20	T1N0M0	-
21	T2N0M0	-
22	T3N0M0	-
23	T3N0M0	-
24	T2N0M0	-
25	T2N0M0	+
26	T2N0M0	+
27	T2N0M0	+
28	T2N0M0	-
29	T2N0M0	-
30	T2N0M0	+
31	T2N0M0	-
32	T2N0M0	+
33	T3N0M0	+
34	T2N0M0	-
35	T2N0M0	+

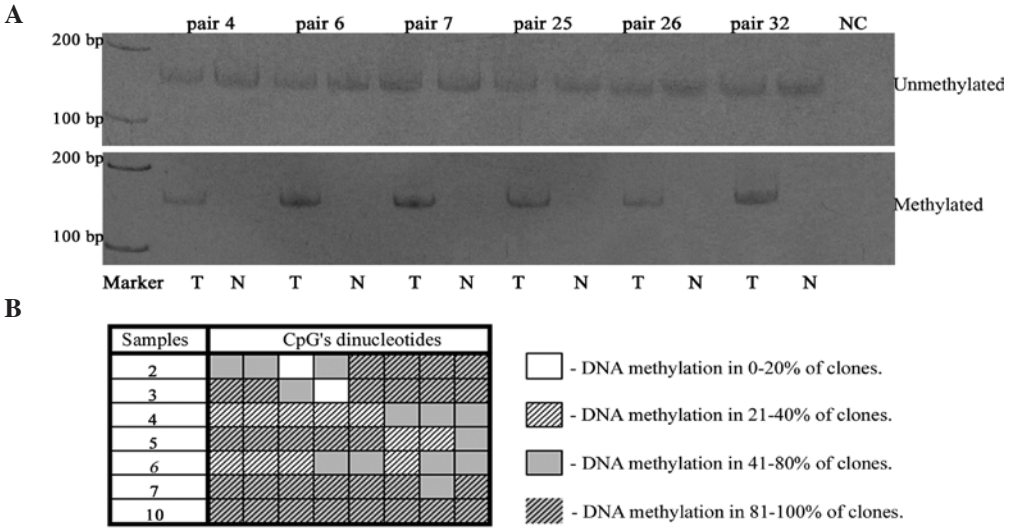

Figure 1. Results of the methylation analysis (A) using methyl-specific PC and (B) MSP-sequence assay of the *LRRC3B* gene promoter region. M, marker; T, tumor sample; N, normal sample; NC, negative control. PCR, polymerase chain reaction; MSP, methyl-specific PCR.

Table II. Bisulfite sequencing results for the *Not*I site surrounding the region of the *LRRC3B* gene.

Sample and ratio (T/N)	Clones	Number of methylated CpG's
T1	1	0
(0.55)	2	6, 22, 35, 44, 48
	3	All CpG's
	4	5, 34
	5	28, 32
	6	6, 22, 35, 49
T2	1	2, 4, 27, 32, 40, 51
(0.49)	2	8, 14
	3	0
	4	0
	5	1, 3, 8, 28, 29, 30, 35, 41, 51
	6	17, 41
T3	1	0
(0.67)	2	30, 34, 35, 44, 46
	3	32, 33, 34, 35, 37
	4	22, 41, 52
	5	46, 47, 53, 54
	6	0

ylated *LRRC3B* gene contained 8 CpG-dinucleotides. The results of MSP-sequencing assay for the *LRRC3B* gene are presented in Fig. 1B.

To confirm these results, the bisulfite sequencing of the *LRRC3B* promoter region of three tumor samples and the corresponding adjacent non-malignant renal tissue was performed. The results of bisulfite sequencing are presented in Table II. We detected both the methylated and unmethylated clones in tumors, while only the unmethylated clones were detected in normal tissues.

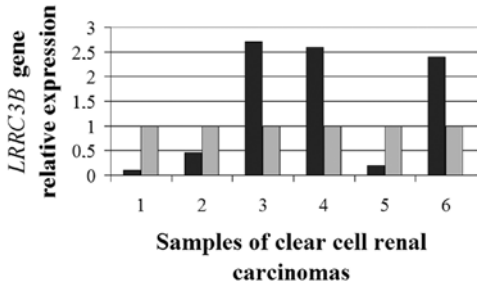

Figure 2. Relative expression of the *LRRC3B* gene in clear cell renal cell carcinoma samples. Grey bars, adjacent non-malignant renal tissues; black bars, clear cell renal cell carcinomas.

To find a correlation between methylation of the promoter region and expression of *LRRC3B*, q-PCR was used in 6 methylated samples. We found that the *LRRC3B* gene was downregulated in 3 samples (≤0.5-fold) and significantly upregulated in another 3 samples (~2.5-fold) (Fig. 2). Therefore, we conclude that there is no clear correlation between *LRRC3B* expression and its promoter methylation.

Discussion

The *LRRC3B* gene is a putative tumor-suppressor gene that is located in the 3p24 region of human chromosome 3. It spans approximately 88 kbp and consists of 2 exons (http://www.ncbi.nlm.nih.gov/gene/116135). A product of the *LRRC3B* gene is a member of the transmembrane protein family, containing a leucine-rich repeat (LRR)-domain (17). These proteins have essential functions in various processes, including the regulation of mRNA decay during cell cycle progression and prevention of cell death and senescence (18). It has been suggested that the LRRC3B protein may regulate expression of the genes encoding components of the DNA damage checkpoint and repair pathways. It was also found that repair was more rapidly completed and more efficient in *LRRC3B*-positive cells following ultraviolet (UV) exposure. Moreover, it has

been shown that the level of cyclin B1 was decreased in HeLa cells following re-expression of *LRRC3B* (19). Noteworthy, the elevated level of expression of genes that are involved in the immune response and interferon (IFN) pathways was detected in xenograft tumors that were derived from a cell line that constitutively expressed *LRRC3B* (15).

It has been shown that DNA methylation of the promoter regions of tumor-suppressor genes plays a crucial role in cancer development. The common mechanism of *LRRC3B* inactivation is methylation of its 5'-CpG-islands. Hypermethylation of the 5'-CpG-island of the *LRRC3B* gene was detected in various types of cancer, including colon (20) and gastric (15) tumors, as well as acute leukaemia (21,22).

We previously showed, using NotI-microarray, that the *LRRC3B* gene is frequently affected in various types of tumors (renal, ovary and colon) owing to genetic (deletions or amplifications) and epigenetic (promoter methylation/demethylation) changes (12,23,24). To assess the mechanism of *LRRC3B* alterations in clear cell RCCs, an analysis of DNA methylation of the *LRRC3B* promoter region was performed using MSP. We found that the promoter region of *LRRC3B* is hypermethylated in clear cell RCCs. DNA methylation of the *LRRC3B* gene was detected in 43% (15 out of 35) of cases. Therefore, the results of the MSP assay are in concordance with the NotI-microarray data that were previously obtained in our laboratory. NotI-microarray techniques allow us to detect genetic and epigenetic alterations simultaneously. However, we were unable to draw conclusions on the genetic or epigenetic causes of *LRRC3B* gene inactivation (9). MSP precisely detects DNA methylation. Therefore, we propose that the main alteration of the *LRRC3B* gene is its promoter methylation. A sequencing of MSP products confirmed the methylation-specific PCR data.

We selected a set of clear cell RCC samples where the *LRRC3B* promoter was methylated, as detected by MSP assay. q-PCR did not reveal a correlation between the methylation of the *LRRC3B* promoter and its expression levels. Bioinformatic analysis of EST-database detected the isoforms of *LRRC3B* mRNA that were not discussed earlier. Possible usage of other promoters may explain an absence of the correlation between CpG-island methylation and level of expression of the *LRRC3B* gene.

In conclusion, our results reveal that the 5'-CpG islands in the LRRC3B locus are frequently hypermethylated in clear cell renal cell carcinomas. However, no correlation between DNA methylation of the 5'-CpG island and expression of the *LRRC3B* gene was found in clear cell RCCs.

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References

1. Jemal A, Siegel R, Xu J and Ward E: Cancer statistics, 2010. Cancer J Clin 60: 277-300, 2010.
2. Eble JN, Sauter G, Epstein JI, *et al* (eds): Pathology and Genetics of Tumors of theUrinary System and Male Genital Organs. World Health Organization Classification of Tumors. p7, 2004.
3. Arai E and Kanai Y: Genetic and epigenetic alterations during renal carcinogenesis. Int J Clin Exp Pathol 4, 58-73, 2011.
4. Crossey PA, Foster K, Richards FM *et al*: Molecular genetic investigations of the mechanism of tumorigenesis in von Hippel-Lindau disease: analysis of allele loss in VHL tumors. Hum Genet 93: 53-58, 1994.
5. Li J, Wang F, Haraldson K, *et al*: Functional characterization of the candidate tumor-suppressor gene NPRL2/G21 located in 3p21.3C. Cancer Res 64: 6438-6443, 2004.
6. Kashuba VI, Li J, Wang F, *et al*: RBSP3 (HYA22) is a tumor suppressor gene implicated in major epithelial malignancies. Proc Natl Acad Sci USA 101: 4906-4911, 2004.
7. Klein G, Imreh S and Zabarovsky ER: Why do we not all die of cancer at an early age? Adv Cancer Res 98: 1-16, 2007.
8. Morrissey C, Martinez A, Zatyka M, *et al*: Epigenetic inactivation of the RASSF1A 3p21.3 tumor suppressor gene in both clear cell and papillary renal cell carcinoma. Cancer Res 61: 7277-7281, 2001.
9. Ying J, Poon FF, Yu J *et al*: DLEC1 is a functional 3p22.3 tumor suppressor silenced by promoter CpG methylation in colon and gastric cancers. Br J Cancer 100: 663-669, 2009.
10. Kvasha S, Gordiyuk V, Kondratov A, Ugryn D, Zgonnyk YM, Rynditch AV and Vozianov AF: Hypermethylation of the 5'CpG island of the FHIT gene in clear cell renal carcinomas. Cancer Lett 265: 250-257, 2008.
11. Dreijerink K, Braga E, Kuzmin I, *et al*: The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc Natl Acad Sci USA 98: 7504-7509, 2001.
12. Skrypkina IYa, Kashuba VI, Gordiyuk VV, *et al*: Identification of changes in gene loci potentially associated with renal cancer by novel technique of NotI microarrays. Ukr Biokhim Zh 11: 188-192, 2006
13. Sambrook J, Fritsch EF and Maniatis T (eds): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, NY, p181, 1989.
14. Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826, 1996.
15. Spiess AN, Feig C and Ritz C: Highly accurate sigmoidal fitting of real-time PCR data by introducing a parameter for asymmetry BMC Bioinformatics 9: 221-233, 2008.
16. Kim M, Kim JH, Jang HR, *et al*: LRRC3B, encoding a leucine-rich repeat-containing protein, is a putative tumor-suppressor gene in gastric cancer. Cancer Res 68: 7147-7155, 2008.
17. Clark HF, Gurney AL, Abaya E, *et al*: The secreted protein discovery initiative (SPDI), a large-scale effort to identify novel human secreted and transmembrane proteins: a bioinformatics assessment. Genome Res 13: 2265-2270, 2003.
18. Mittal S, Aslam A, Doidge R, Medica R and Winkler GS: The Ccr4a (CNOT6) and Ccr4b (CNOT6L) deadenylase subunits of the human Ccr4-Not complex contribute to the prevention of cell death and senescence. Mol Biol Cell 22: 748-758, 2011.
19. Xu ZM, Gao WR, Mei Q, Chen J and Lu J: The novel gene LRP15 is regulated by DNA methylation and confers increased efficiency of DNA repair of ultraviolet-induced DNA damage. BMB Rep 41: 230-235, 2008.
20. Tian XQ, Zhang Y, Sun D, Zhao S, Xiong H and Fang J: Epigenetic silencing of LRRC3B in colorectal cancer. Scand J Gastroenterol 44: 79-84, 2009.
21. Dou LP, Wang C, Xu ZM, Kang HY, Fan H, Lou FD and Yu L: Methylation pattern of LRP15 gene in leukemia. Chin Med Sci J 22: 187-191, 2007.
22. Dunwell TL, Hesson LB, Pavlova T, *et al*: Epigenetic analysis of childhood acute lymphoblastic leukemia. Epigenetics 4, 185-193, 2009.
23. Gordiyuk VV, Gerashchenko GV, Skrypkina IYa, *et al*: Identification of chromosome 3 epigenetic and genetic abnormalities and gene expression changes in ovarian cancer. Biopolymers Cell 24: 323-332, 2008.
24. Gerashchenko GV, Gordiyuk VV, Skrypkina IYa *et al*: Screening of epigenetic and genetic disturbances of human chromosome 3 genes in colorectal cancer. Ukr Biokhim Zh 81: 81-87, 2009