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ORIGINAL RESEARCH ARTICLE

Anomalies in MiRNAs Machinery Gene, GEMIN-4 Variants Suggest Renal Cell Carcinoma Risk: A Small Experimental Study from North India

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Abstract *GEMIN4* is a member of the *GEMIN* gene family which is involved in multiple pathologies including cancer. It is located on Chr17p13.3, the most notorious chromosome and a hotspot for various carcinomas. We therefore intend to find genetic variants of *GEMIN4* gene associated with renal cell carcinoma risk (RCC). This study comprised 100 patients and 225 controls. Genotyping of *GEMIN4* gene variants was done using Taqman® assay. The association of *GEMIN4* variants and risk prediction of RCC was done by statistical analysis. Haplotype analysis was done to see the combined effect of variants on RCC. Patients carrying variant genotype, CC of *GEMIN4* T/C rs7813 showed significant association whereas in case of *GEMIN4* G/C rs910925 variant genotype, CC significant risk was found. *GEMIN4* rs7813 T/C variant genotype, CC showed risk with smoking ($p = 0.034$). Our study gives a substantive support for the association between the *GEMIN4* gene variants and RCC risk.

Keywords Renal cell carcinoma (RCC) · *GEMIN4* gene · Cancer susceptibility

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Introduction

Renal cell carcinoma (RCC) is the third most common genitourinary malignancies. RCC accounts for more than 87% of all renal malignancies and is the most common neoplasm of the kidney. Renal carcinoma is heterogeneous being comprised of variety of histological variants having different survivals, genetics and response to the treatment given. The most common pathology for RCC is clear cell renal cell carcinoma (ccRCC). The rate of occurrence of RCC varies worldwide being the highest in Europe and North America and lowest in Asia and South America [1]. The ratio for RCC occurrence M:F is 2:1 [1]. The precise cause of RCC is poorly understood, but some specific lifestyle factors such as smoking can play an important etiological factor.

MicroRNAs (miRNAs) are about 20 nucleotide long non-coding RNA molecules which plays a role in post-transcriptional regulation [2, 3]. MiRNAs play a regulatory role in many human genes. Any misregulation in post transcriptional events could lead to cancer occurrence and progression [4]. Some studies reported the association of pre-miRNA gene variants to prostate cancer, supporting the potential interdependence between alterations in the miRNA pathway and the development of prostate cancer [5].

Gemin4 protein is a key member of GEMIN protein family which is involved in various physiological processes [6]. SNPs in the *GEMIN4* gene affects DNA repair in liver cancers and hence lead to the development of liver cancers in Chinese population [7]. Studies have shown association of *GEMIN4* SNPs with the clinical outcome of bladder cancer [8], renal cancer [9, 10], ovarian cancer [11], lung cancer [12], prostate cancer [6] and esophageal cancer [13]. Any truncation of Gemin4 protein will affect the

differential expression of some miRNAs which could be related to the malignant tumors. There is no consensus about the effects of *GEMIN4* gene variants in the pathogenesis of RCC among North Indians. Therefore, the present pilot study is designed to evaluate the association between common polymorphisms in the *GEMIN4* gene and the risk of RCC in North Indian population.

Materials and Methods

Ethical Statement

The study design was approved by the Institutional Ethics Committee. Duly signed informed consents were taken from each study subject.

Study Subjects

The present study of RCC was conducted in the Department of Urology and Renal Transplantation. 100 histologically confirmed RCC patients (M/F 81/19; mean age 49.93 + 11.39 years) were enrolled for the study. Exclusion criteria for patient selection was chemotherapy and radiation therapies. 225 unrelated controls (mean age 55.08 years, and M/F ratio as 202/23) were recruited randomly from the unrelated individuals. 6 ml of peripheral blood was collected from every patient with 3 ml of blood each in different vials containing 0.5 M EDTA (pH 8.0) as an anti-coagulant and stored at − 80 °C for further analysis. At the time of enrolment patients and controls were interviewed for demographic details and lifestyle details like smoking. The RCC patients were grouped further into non-smokers and smokers.

Clinical Data Collection

The basic detail and characteristics of patients are demonstrated in Table 1. The pathological details of the tumor and therapy, relapse etc. were provided by concerned personnels. American Joint Committee on Cancer's TNM staging system was used to classify the tumor. 79 of 100 patients had clear cell RCC (ccRCC) type and rest had non-clear cell RCC.

SNPs Selected

SNPs were selected based on available literature and their role in various carcinomas. SNP info and F-SNP were used for Tag SNPs selection. Three SNPs of *GEMIN4* viz. *GEMIN4* C/T rs3744741, *GEMIN4* T/C rs7813 and *GEMIN4* G/C rs910925 were selected for the present study. Details of selected SNPs are given in Table 2.

Table 1 Demographical details of renal cell cancer patients and healthy controls

Variables	Patients n = 100 n (%)	Controls n = 225 n (%)	#*p* value
Sex			
Female	19 (19.0)	23 (10.2)	0.229
Male	81 (81.0)	202 (89.8)	
Age (years)			
Mean age ± SD	49.93 ± 11.39	55.08 ± 10.14	0.313
Smoking[a]			
Non-smokers	32 (34.4)	161 (73.9)	< **0.001**
Smokers	61 (65.6)	57 (26.1)	
Stage			
T1	25 (25.0)	–	–
T2	43 (43.0)	–	
T3	12 (12.0)	–	
T4	20 (20.0)		
Grade			
G1	20 (20.0)	–	–
G2	42 (42.0)	–	
G3	38 (38.0)	–	
Histology			
Clear cell RCC	79 (79.0)		
Non-clear cell RCC	21 (21.0)		

The statisticaly significant values are shown in bold

BCG i + m Bacillus Calmette-Guerin induction + maintenance

[a]The sum could not add up to the total due to some missing values

[#]Student *t* test was used to determine the *p* value

Table 2 Characteristic of candidate SNPs of *GEMIN-4 Gene*

SNP ID	Major/minor allele	MAF (%)
rs3744741	C/T	28
rs7813	T/C	29
rs910925	G/C	29

SNP single nucleotide polymorphism, *MAF* minor allele frequency

DNA Extraction and Genotyping

Blood DNA was extracted using salting-out method [14]. Polymorphisms in *GEMIN4* C/T rs3744741, *GEMIN4* T/C rs7813 and *GEMIN4* G/C rs910925 were genotyped using TaqMan SNP (Applied Biosystems, USA) genotyping assay using 96-well plate Real time PCR system along with positive and negative controls each assay, and 10% of the samples were randomly selected and run in duplicates with 100% concordance.

Statistical Analysis

The power of the study was calculated using Quanto software, version 1.0 (available from: http://hydra.usc.edu/gxe) To analyse basal characteristics, we used Chi square tests for the categorical data. Associations between *GEMIN4* gene polymorphisms and RCC risk were estimated using adjusted odds ratios (AORs) and 95% confidence intervals (95% CIs) from multivariate logistic regression, which was used to adjust the effect factor (i.e., age, gender and smoking). The statistical analysis was done using the Statistical Package for Social Sciences software, version 20.0 (SPSS, Chicago, IL), and $p < 0.05$ was considered statistically significant.

In Silico Analysis

Functional effects in *GEMIN4* gene was determined using F-SNP (http://compbio.cs.queensu.ca/F-SNP/) [15].

Results

Demographic Details

The characterization of patients and controls are demonstrated in Table 1. There was no significant difference between the patients and controls on age ($p = 0.313$) and gender ($p = 0.229$). Number of patients with smoking habit (65.6%) are higher than controls (p value $= 0.001$). 79% of patients had conventional clear cell carcinoma. Patients having other histological forms of carcinoma were 21%. Approximately 25% of patients were in stage I, 43, 12, and 20% was found to be in stage II, III, and IV, respectively. Genotype distributions for all the SNPs in the control group were in concordance with Hardy–Weinberg equilibrium ($p = 0.855$).

Genotyping and Association of *GEMIN4* C/T rs3744741, *GEMIN4* T/C rs7813 and *GEMIN4* G/C rs910925 with RCC

The gene variants of *GEMIN4* viz. *GEMIN4* C/T rs3744741, *GEMIN4* T/C rs7813 and *GEMIN4* G/C rs910925 were evaluated and their genotype distribution in RCC patients is detailed in Table 3. No significant association of *GEMIN4* C/T rs3744741 was seen in case of RCC risk (CT type; OR 1.18, CI 0.65–2.14, $p = 0.582$, TT type; OR 2.16, CI 0.85–5.53, $p = 0.106$). We did not find any association in the dominant model (CT + TT; OR 1.31, CI 0.75–2.28, $p = 0.339$) or at allelic level (T allele; OR 1.26, CI 0.88–1.82, $p = 0.206$) of *GEMIN4* C/T rs3744741 in RCC. Although the other two variants of *GEMIN4* were found to be associated with manifold risk in developing RCC. In case of *GEMIN4* T/C rs7813, significant association was observed in variant genotype (CC type; OR 3.37, CI 1.44–7.89, $p = 0.005$), dominant model (TC + CC; OR 1.94, CI 1.11–3.41, $p = 0.021$) as well as at allelic level in variant allele (C allele; OR 1.66, CI 1.17–2.35, $p = 0.004$). While in the third variant of *GEMIN4*, i.e. *GEMIN4* G/C rs910925 significant risk was seen only in variant genotype (CC type; OR 2.37, CI 1.12–4.99, $p = 0.024$) and variant allele (C allele; OR 1.56, CI 1.11–2.19, $p = 0.010$).

Association of *GEMIN4* Genotypes with Smoking as a Risk Factor of RCC

After analysing the data at genotypic and allelic level we tried to relate the association with smoking, which is a great risk factor in causing RCC. For this study, we stratified the patients amongst two groups: smokers and non-smokers, and calculations were done accordingly. We found significant association in variant genotype of *GEMIN4* T/C rs7813 with smoking in RCC (CC; OR 3.51, CI 1.16–4.29, $p = 0.034$). Whereas, the other gene variants *GEMIN4* C/T rs3744741 and *GEMIN4* G/C rs910925 did not show any association with smoking risk factor in RCC. The detailed data is shown in Table 4.

Association of Histological Cell Type of RCC with *GEMIN4* Gene Variants

Tumor histology plays a major role in detection of cancer stage as well as grade. If any gene is associated with the cellular histology of tumor tissue, we can detect the stage by detecting that gene. Here we tried to correlate the *GEMIN4* gene variants at genotypic level with RCC cell type to find any significant association. No significant association of any of the three gene variants of *GEMIN4* with RCC risk was observed. No association of RCC tumor with *GEMIN4* gene variants could be due to small number of samples. (Table 5).

Association of *GEMIN4* C/T rs3744741, *GEMIN4* T/C rs7813 and *GEMIN4* G/C rs910925 Haplotypes with RCC Risk

Haplotype analysis (Combinatorial effect of SNPs) may be more manifesting in predicting the association compared to single nucleotide polymorphism, as individual polymorphism is likely to confer modest effects to the risk of RCC. We constructed haplotype sets for *GEMIN4* gene polymorphisms, wherein CTG was taken as a reference. We demonstrate significant association in 3 sets of haplotype

Table 3 Genotype and allelic frequency distribution of *GEMIN4 C/T (rs3744741), GEMIN4 T/C (rs7813)* and *GEMIN4 G/C* (*rs910925)* gene polymorphism in RCC patient and healthy controls

Genetic model	Genotypes	Controls n = 225 n (%)	Patients n = 100 n (%)	#*p* value	OR[a] (95%CI)
GEMIN4 C/T rs3744741					
Additive	CC	123 (54.7)	51 (51.0)	Ref	Ref
	CT	86 (38.2)	36 (36.0)	0.582	1.18 (0.65–2.14)
	TT	16 (7.1)	13 (13.0)	0.106	2.16 (0.85–5.53)
Dominant	CC	123 (54.7)	51 (51.0)	Ref	Ref
	CT + TT	102 (45.3)	49 (49.0)	0.339	1.31 (0.75–2.28)
Multiple	C	332 (73.8)	138 (69.0)	Ref	Ref
	T	118 (26.2)	62 (31.0)	0.209	1.26 (0.88–1.82)
GEMIN4 T/C rs7813					
Additive	TT	115 (51.1)	39 (39.0)	Ref	Ref
	TC	89 (39.6)	41 (41.0)	0.918	1.03 (0.56–1.90)
	CC	21 (9.3)	20 (20.0)	**0.005**	3.37 (1.44–7.89)
Dominant	TT	115 (51.1)	39 (39.0)	Ref	Ref
	TC + CC	110 (48.9)	61 (61.0)	**0.021**	1.94 (1.11–3.41)
Multiple	T	319 (70.9)	119 (59.5)	Ref	Ref
	C	131 (29.1)	81 (40.5)	**0.004**	1.66 (1.17–2.35)
GEMIN4 G/C rs910925					
Additive	GG	100 (44.4)	37 (37.0)	Ref	Ref
	GC	93 (41.3)	35 (35.0)	0.693	0.88 (0.47–1.65)
	CC	32 (14.2)	28 (28.0)	**0.024**	2.37 (1.12–4.99)
Dominant	GG	100 (44.4)	37 (37.0)	Ref	Ref
	GC + CC	125 (55.5)	63 (63.0)	0.062	1.72 (0.97–3.04)
Multiple	G	293 (65.1)	109 (54.5)	Ref	Ref
	C	157 (34.9)	91 (45.5)	**0.010**	1.56 (1.11–2.19)

The statistically significant values are shown in bold

OR odds ratio, *CI* confidence interval

[a]OR (95%CI); Age, gender and smoking adjusted odds ratio and 95% confidence interval

[#]Student's *t* test was used to determine *p* value

combinations (TTG, CCC and TCC) with RCC risk, the significant *p* values and OR are shown in Fig. 1.

In silico Analysis for the Functionality of GEMIN-4 Gene Variants

The location of SNPs of *GEMIN4* gene i.e. rs3744741, rs7813 and rs910925 is described in Table 6 and the results of In-silico analysis showed change in transcriptional regulation for all the candidate SNPs (Table 6).

Discussion

GEMIN4 gene is located on chromosome 17p13.3, a hotspot for various melanomas, and is a protein coding gene. *GEMIN4* is an important gene of miRNA machinery. The expression of many human genes is regulated by miRNA processing. [6, 16]. Aberrations in the regulatory pathway of the miRNA could lead to altered miRNA transcription, splicing, and transcriptional regulation of cell proliferative and apoptotic genes. Therefore, polymorphisms in the miRNA pathways may contribute to cancer progression [5]. In this study, significant associations were found in SNPs of *GEMIN4* gene and RCC risk. The results of this study would lead to the further concept of the mechanism of expression of miRNAs and their relation with genetic variants in the miRNA regulatory pathway genes viz. *GEMIN4* and the susceptibility of RCC. Our findings are consistent with the results that these two polymorphisms, *GEMIN4* G/C rs910925 and *GEMIN4* T/C rs7813, have potential roles in carcinogenesis, such as renal cell carcinoma [10], bladder cancer [9], and ovarian cancer [17] in other studies. No significant association with the third polymorphism and RCC risk was observed in our study, suggestive of either no role in the pathogenesis of RCC, or

Table 4 Association of *GEMIN4 C/T (rs3744741), GEMIN4 T/C (rs7813)* and *GEMIN4 G/C* (rs*910925)* gene variants with smoking habit in RCC susceptibility

Genotype	Patients Non smokers n = 32; n (%)	Patients Smoker n = 61; n (%)	*p* value	OR[a] (95% CI)
GEMIN4 C/T rs3744741				
CC	16 (50.0)	30 (49.2)	**Ref**	**Ref**
CT	10 (31.2)	24 (39.3)	0.721	0.80 (0.23–2.71)
TT	6 (18.8)	7 (11.5)	0.129	0.31 (0.07–1.41)
GEMIN4 T/C rs7813				
TT	11 (45.8)	35 (50.7)	**Ref**	**Ref**
TC	8 (33.4)	26 (37.7)	0.227	2.09 (0.63–6.91)
CC	5 (20.8)	8 (11.6)	**0.034**	3.51 (1.16–4.29)
GEMIN4 G/C rs910925				
GG	9 (37.5)	26 (37.7)	**Ref**	**Ref**
GC	7 (29.2)	25 (36.2)	0.507	1.53 (0.43–5.42)
CC	8 (33.3)	18 (26.1)	0.587	1.46 (0.37–5.85)

Statistically significant values are shown in bold

OR odds ratio, *CI* confidence interval

[a]OR (95%CI) Age, gender adjusted odds ratio and 95% confidence interval

Table 5 Genotype frequency of *GEMIN4 C/T (rs3744741), GEMIN4 T/C (rs7813)* and *GEMIN4 G/C* (rs*910925)* of RCC patients based on RCC cell type

Genotype	Cell type		*p* value	OR[a] (95%CI)
	Non-clear RCC (n = 21)	Clear cell RCC (n = 79)		
GEMIN4 C/T rs3744741				
CC	12 (57.1)	39 (49.4)	Ref	Ref
CT	6 (28.6)	30 (38.0)	0.483	0.650 (0.219–1.932)
TT	3 (14.3)	10 (12.7)	0.973	0.975 (0.230–4.129)
GEMIN4 T/C rs7813				
TT	9 (42.9)	30 (38.0)	Ref	Ref
TC	9 (42.9)	32 (40.5)	0.904	0.938 (0.328–2.678)
CC	3 (14.3)	17 (21.5)	0.469	0.588 (0.140–2.472)
GEMIN4 G/C rs910925				
GG	9 (42.9)	28 (35.4)	Ref	Ref
GC	7 (33.3)	28 (35.4)	0.660	0.778 (0.254–2.379)
CC	5 (23.8)	23 (29.1)	0.531	0.676 (0.199–2.301)

[a]OR (95%CI) Age, gender adjusted odds ratio and 95% confidence interval

OR odds ratio, *CI* confidence interval

perhaps related to the limited sample size. So, case–control studies with large sample size and of different ethnicity should be performed to validate our results. In this study, we found significant associations between SNPs in miRNAs biogenesis pathway and the risk of RCC. Recent studies have shown that, disrupting miRNAs processing through the knockdown of DROSHA, DGCR8, and DICER1, could accelerate cellular transformation and tumorigenesis [8].

Thomson et al. [18] have shown that the repression of mature miRNAs is not consistent with the reductions in the primary miRNAs transcripts, suggesting the existence of altered regulations of miRNAs processing in human cancers. These lines of evidence are in concordance with the recent profiling of miRNAs expression, which showed the general repression of miRNAs in a variety of tumors and cancer cell lines [6, 8, 16].

The findings of our study indicate that genetic alterations of miRNAs regulation might be associated with cancer development and progression. We analyzed the haplotype combination of *GEMIN4* gene variants with their three polymorphic sites and RCC risk. We found significant association in three sets with more than 1.5 folds' risk in RCC. Three SNPs of the *GEMIN4* (rs7813 and

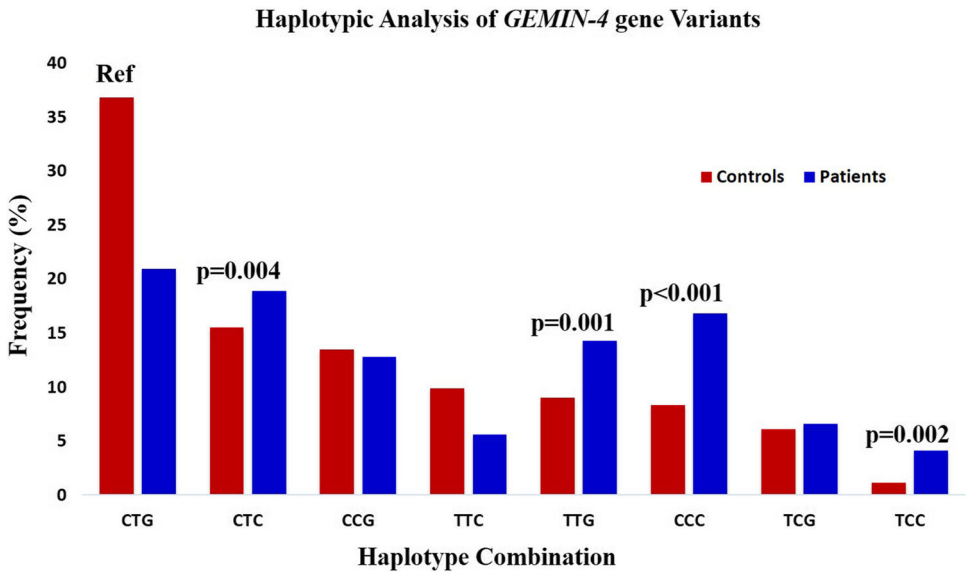

Fig. 1 Haplotypic analysis of *GEMIN4 gene* variants in RCC patients and controls

Table 6 In silico analysis of *GEMIN4* gene polymorphisms by F-SNP

SNPs of *GEMIN-4* gene	Functional category	Prediction tool	Prediction result	FS score	Location
rs3744741	Protein coding	SNP effect	Deleterious	1	Exonic
	Splice regulation	ESE finder	Changed		
	Post translational	OGPET	Exist		
rs7813	Protein coding	SNP effect	Deleterious	0.451	3′UTR
	Splice regulation	ESE finder	Changed		
	Post translational	OGPET	Not exist		
rs910925	Protein coding	SNP effect	Benign	0.264	Exonic
	Splice regulation	ESE finder	Changed		
	Post translational	OGPET	Not exist		

rs2740348) and *GEMIN3* gene (rs197412) were found to be associated with altered RCC risk [10].

Nonetheless, we tried to get more powerful comprehension about the influence of these SNPs on RCC risk using a pathway-based polygenic approach and identify a pattern toward an increasing RCC risk with an increasing number of unfavourable genotypes. Yang and group in one of their study have discussed about genetic variants in miRNA genes and their biogenesis pathway and their susceptibility to bladder cancer. In the same study, they have elaborated about 41 functional miRNA related SNPs, among all, 7 SNPs (rs910924, rs2740348, rs7813, rs910925, rs3744741, rs1062923, rs4968104) of GEMIN-4 gene were discussed [8]. One SNP of *GEMIN-4* gene i.e. rs7813 was found to be significantly associated with bladder cancer risk [8], hepatocellular carcinoma [7], which complies to our results. Yang et al. [8], also found the SNPs to have physiological effects and tumorigenesis of bladder cancer. F-SNP is a collection of all the SNPs having functional effects on various carcinomas. The functional effects of *GEMIN4* C/T rs3744741, *GEMIN4* T/C rs7813 and *GEMIN4* G/C rs910925 is discussed in Table 6, rs3744741 and rs7813 have deleterious effect in bladder cancer indicating a predisposition of these SNPs in risk of bladder cancer (http://compbio.cs.queensu.ca/F-SNP/).

This finding thus can contribute to prove the fact that RCC is polygenic process and thus miRNAs machinery genes may have a greater ability to characterize high-risk populations. Further studies in a larger population are required to validate these results. The *GEMIN-4* gene, one of the miRNAs regulatory pathway genes, contributes to increased risk of RCC.

Limitations of the Study

The sample size of our study was relatively small; therefore, studies with larger sample sizes with sufficient large subgroups are warranted to validate our findings.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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