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Wnt antagonist gene polymorphisms and renal cancer

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Abstract

Purpose—Epigenetic silencing of several Wnt pathway related genes has been reported in renal cancer. Except for the TCF4 gene, there are no reports regarding Wnt pathway gene polymorphisms in renal cancer. Therefore, we hypothesized that the polymorphisms in Wnt signaling genes may be risk factors for renal cancer.

Experimental Design—A total of 210 patients (145 male and 65 female) with pathologically confirmed renal cell carcinoma (RCC), and 200 age- and sex-matched control individuals were enrolled in this study. We genotyped 14 SNPs in six genes including *DKK2* (rs17037102, rs419558, rs447372), *DKK3* (rs3206824, rs11022095, rs1472189, rs7396187, rs2291599), *DKK4* (rs2073664), *sFRP4* (rs1802073, rs1802074), *SMAD7* (rs12953717), *DAAM2* (rs6937133, rs2504106) using PCR-RFLP and direct sequencing in RCC and age-matched healthy subjects. We also tested the relationship between these polymorphisms and clinicopathologic data including gender, grade, tumor stage, lymph-node involvement, distant metastasis, and overall survival.

Results—A significant decrease in the frequency of the G/A+A/A genotypes in the *DKK3 codon335* rs3206824 was observed in RCC patients compared with controls. The frequency of the rs3206824 (G/A) A- rs7396187 (G/C) C haplotype was significantly lower in RCC compared with other haplotypes. We also found that *DKK3* rs1472189 C/T is associated with distant metastasis and furthermore, *DKK2* rs17037102 G homozygous patients had a decreased risk for death by multivariate Cox regression analysis.

Conclusions—This is the first report documenting that *DKK3* polymorphisms are associated with RCC and that the *DKK2* rs17037102 polymorphism may be a predictor for survival in RCC patients after radical nephrectomy.

Keywords

Polymorphism; Wnt; DKK; sFRP; RCC

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INTRODUCTION

Renal cell carcinoma (RCC) is the third leading cause of death among urological tumors, accounting for about 2% of adult malignancies.[1] Although, the rate of detection of RCC has increased with improved diagnostic techniques, metastatic lesions are still found at diagnosis in about 25% of RCC patients. Moreover, in RCC patients, distant metastases are sometimes found long after surgical removal of the primary tumor. After detecting these metastases, the 5 years survival rate is generally less than 10%.[2] The standard treatment for localized renal cancer is surgical removal,[2] while immunotherapy is used for metastatic disease because of its multi-drug resistance. Interleukin (IL)-2 is the most common immunotherapy for RCC but is effective in only 10 to 15% of patients.[3]

Wnt/β-catenin signaling is involved in numerous processes in development, is strongly implicated in tumorigenesis and highly related to tumor invasion and metastasis.[4]

Wnt signals transduced by the canonical pathway play a role in determining cell fate and those by the noncanonical pathway are important for control of cell movement and tissue polarity.[5]

Canonical Wnt ligands bind to frizzled (FZD) family receptors and the LRP5/LRP6 co-receptor, which stabilize β-catenin. Subsequently β-catenin interacts with members of the lymphoid enhancer factor 1/T-cell factor (LEF1/TCF) family, resulting in generation of a functional transcription factor complex and the expression of downstream target genes. [5] Non-canonical Wnt ligands bind to FZD family receptors, and ROR2 and RYK co-receptors.[5–7] This signaling is mainly involved in cytoskeletal reorganization during cancer cell invasion and metastasis.[6][7] At present, five Wnt antagonist families have been described, namely, secreted frizzled-related protein (sFRP), Wnt inhibitory factor 1 (Wif1), Xenopus Cerberus, Wise and Dickkopf (DKK) families.[8]

The sFRP family (sFRP1-sFRP5) and Wnt inhibitory factor-1 (Wif-1) are involved in inhibiting Wnt signaling by directly binding to Wnt molecules.[8] The DKK family (DKK1-DKK4) inhibits Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex.[8]

Transforming growth factor-β (TGF-β) also regulates cell fate and proliferation co-operatively with Wnt proteins.[9][10] The Smad proteins transduce signaling from the activated TGF-β –TGF receptor complex.[11] Smad proteins, together with β–catenin, act as a transcriptional regulator in the TGF-β and Wnt signaling pathways.[11]

Epigenetic silencing of several Wnt pathway genes has been reported in renal cancer. [12–15] However, there have been no reports regarding Wnt pathway related gene polymorphisms in renal cancer except for the TCF4 gene.[16]

Therefore, with this evidence, we hypothesized that gene polymorphisms associated with Wnt (canonical and noncanonical) and TGF-β cascades may be associated with renal cancer risk. To test this hypothesis, we conducted a case-control study with respect to 14 single nucleotide polymorphisms (SNPs) including *DKK2* (rs17037102, rs419558, rs447372), *DKK3* (rs3206824, rs11022095, rs1472189, rs7396187, rs2291599), *DKK4* (rs2073664), *sFRP4* (rs1802073, rs1802074), *DAAM2* (rs6937133, rs2504106), and *SMAD7* (rs12953717). We selected these polymorphic sites based on previous reports and HapMap data (http://www.hapmap.org/),[17][18] which are composed of possibly functional (non-synonymous and 5'- or 3'-untranslated region SNPs) or disease-associated SNPs. We then tested the relationship between these polymorphisms and clinicopathologic data including gender, grade, tumor stage, lymph-node involvement, distant metastasis, and overall

survival. We also investigated the relationship between Wnt antagonist genes polymorphisms and the expression of the beta-catenin, which is one of the downstream targets of Wnt signaling.

Materials and Methods

Samples

A total of 210 patients (145 male and 65 female) with pathologically confirmed conventional RCC, and 200 age- and sex-matched control individuals were enrolled in this study. Genomic DNA was extracted from the peripheral blood of 154 patients and 200 healthy individuals (Shimane University Hospital, Izumo, Japan), and from the paraffin-embedded non-cancerous kidney tissue of 56 patients (Toho University Hospital, Tokyo, Japan). A DNA mini kit (Qiagen, Valencia, CA) was used to extract DNA from normal tissue and peripheral blood according to the manufacturers' protocols. The mean ages of the patient and control groups were 62.0 and 61.0 years, respectively (Table 1, $p = 0.35$). All of the patients (n = 210) tested were diagnosed with RCC on the basis of histopathological findings. They were classified according to the WHO criteria and staged according to the tumor-node-metastasis (TNM) classification. Healthy controls consisted of volunteers with no apparent abnormal findings upon medical examination at Shimane University Hospital. These samples are same as previously reported ones. [19]

To ascertain that volunteers were healthy and free of cancer, they all underwent various tests that included physical exams, questionnaires about their health and history, chest X-rays, blood and urine tests for various tumor markers, and abdominal ultrasound, gastric endoscopy, and colon enema. Peripheral blood samples were obtained from the patients and controls after written informed consent was obtained in Shimane University Hospital and Toho University hospital.

Genotyping

Polymorphic sites with minor allele frequencies (MAF) of more than 0.1 in Japanese populations were selected from HapMap data. Information regarding functional polymorphisms (non-synonymous, synonymous, 5'- and 3'- untranslated region,) is added to Table 2 and diagrams of the *DKK2*, *DKK3*, and *sFRP4* genes showing their functional domains and polymorphic sites are in Figure 1. Polymorphisms were analyzed by PCR-RFLP. Genotyping methods, primer sets, and annealing temperatures used for RFLP are shown in Table 2. Each PCR reaction was carried out in a total volume of 20 μL consisting of 0.3 μL of a 10 μmol/L solution of each primer, 1.5 mmol/L $MgCl_2$, 0.8 mmol/L deoxynucleotide triphosphate, 0.5 unit RedTaq DNA polymerase (Sigma), 1 μL of genomic DNA (30 ng/μL), and 15.6 μL H_2O using a PTC 200 Thermal Cycler (MJ Research). All reactions were subjected to two rounds of amplification using a nested primer approach. The first and second PCR annealing temperatures and PCR cycles were 52 °C, 48 cycles, and 58 °C, 45 cycles, respectively. The second PCR products were digested with each restriction enzyme and temperature (New England BioLabs, Waltham, MA) for 3 hours, separated on a 2.0 % agarose gel and subsequently stained with ethidium bromide.

All assays were conducted blindly without the knowledge of case or control status. Two researchers carried out RFLP and reading of the gels. All samples were retested and the results were 100% concordant. To confirm the genotype ascribed by PCR–RFLP, approximately 50% of the PCR sample products were randomly selected and subjected to direct sequencing using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA). There were no discrepancies in the results.

Immunohistochemical study

We performed immunohistochemistry of the Wnt downstream target beta-catenin in formalin-fixed, paraffin-embedded (FFPE) specimens using rabbit polyclonal antibody against human beta-catenin (#9562, Cell signaling Technology, Beverly, MA). The staining procedure was according to a commercial kit (Lab vision, Fremont, CA). We investigated the relationship between Wnt antagonist gene genotypes and beta-catenin expression. The sections were counterstained with Harris' hematoxylin. A typical representative staining is shown in Figure 2.

Statistical analysis

The common homozygote was used as the reference for calculation genotype specific odds ratio. Hazard ratios (HR) and 95% confidence intervals (95% CI) were calculated from the proportional hazard assumption of the Cox regression model including multivariate analysis. The probability of overall survival time was estimated using Kaplan-Meier plots and the log-rank test. Hardy–Weinberg equilibrium and haplotype analysis were evaluated by SNPAlyze version 2.2 (DYNACOM, Tokyo, Japan), utilizing an EM method. The chi-square test was used to compare the genotype frequency between patients and controls. All statistical analyses were performed using StatView (version 5; SAS Institute Inc., NC). We adopted false discovery rate (FDR) according to Benjamini and Hochberg [20][21] and set the statistically significant level as $P < 0.05$. An FDR of 0.05 was used as a critical value for assessment whether the obtained P value was significant. The genotype frequencies of the polymorphisms in control samples ($n = 200$) and case samples (n = 210) did not deviate from Hardy-Weinberg equilibrium ($P > 0.05$).

RESULTS

Comparison of genotype distribution between RCC cases and controls

The genotype distributions of the *DKK2* (rs17037102, rs419558, rs447372), *DKK3* (rs3206824, rs11022095, rs1472189, rs7396187, rs2291599), *DKK4* (rs2073664), *sFRP4* (rs1802073, rs1802074), *SMAD7* (rs12953717), *DAAM2* (rs6937133, rs2504106) polymorphisms between renal cancer cases and healthy controls are shown in Table 3. A significant decrease in the frequency of the G/A + A/A genotypes of *DKK3* rs3206824 (non synonymous Arg335Gly) was observed in renal cancer patients compared with controls (OR = 0.43; 95% CI, 0.29–0.65) (Table 3).

We also found a significant decrease in the frequency of G/C+C/C genotypes of the *DKK3* rs7396187 in patients (OR = 0.54; 95% CI, 0.37–0.81) (Table 3). There was also a significant increased frequency of the *A/A* genotype of rs1802074 SNP of *sFRP4* gene (OR, 2.15; 95% CI, 1.15–4.02), increased frequency of the T/T genotype of rs2073664 of *DKK4* (OR, 2.91; 95% CI, 1.32–6.42), and a significant increase in the T/C + C/C genotypes of *DAAM2* rs2504106 (OR, 0.44; 95% CI, 0.28–0.69) in patients (Table 3). However, no significant difference was observed in the genotype distribution of other genotypes between patients and controls (Table 3).

Next, we investigated gene-gene interaction using *DKK2* rs419558, *DKK2* rs447372, *DKK3* rs3206824, *DKK3* rs7396187, *SMAD7* rs12953717, *sFRP4* rs1802074, and *DAAM2* rs2504106 because we found a significant difference in the genotype distribution between cases and controls. Among these combinations, when the combined effect of 2 polymorphisms (*DKK3* rs3206824 and *sFRP4* rs1802074) was evaluated, a decreased renal cancer risk was found for only the *DKK3* rs3206824 GA + AA and *sFRP4* rs1802074 A/G + G/G genotypes (OR, 0.19; 95% CI, 0.09–0.45; $P < 0.0001$) (Table 4).

Linkage disequilibrium and haplotype analysis in *DKK3* and *DKK2* gene polymorphisms

The *DKK3* rs3206824 polymorphism was in linkage disequilibrium (LD) with rs7396187 (D = 0.1627). Therefore, the frequency of each haplotype including rs3206824 (G/A) and rs7396187 (G/C) was calculated between RCC patients and controls. The frequency of the A–C haplotype was significantly lower in RCC ($P < 0.0001$) compared with other haplotypes (Table 5).

The *DKK2* rs419558 polymorphism was in linkage disequilibrium (LD) with rs447372 (D = 0.2244). However we did not find a significant difference between case and control in the haplotype analysis using rs419558 and rs447372 (Table 5).

Relationship of genotype distribution with clinicopathological characteristics

We investigated the effect of the twelve SNPs on clinicopathological factors including sex, grade, pathological stage (pT), pN, and pM. Regarding gender, pT, and pN, there were no significant effects of SNPs (Table 6). We found a higher frequency of C/T + T/T genotypes in *DKK3* rs1472189 in patients with distant metastasis (pM (+)).

Relationship of various Wnt-antagonist genes genotype with the expression of beta-catenin

We investigated the relationship between the Wnt-antagonist genes genotypes and beta-catenin expression. However, we could not find any relationship between these polymorphisms and beta-catenin expression (Table 7).

Multivariate Cox proportional hazard analysis for overall survival in RCC patients

The prognostic values for overall survival using parameters such as gender, age at diagnosis, tumor grade, pTNM, 14 SNPs were analyzed using Kaplan-Meier survival curves, and it was observed that higher grade (grade 3 + 4), pT3 + pT4, pN (+), pM (+), and *DKK2* rs17037102 G/A+A/A genotypes were associated with shorter survival (Fig. 3).

In addition to pT3 + pT4, pN (+) and pM (+), the *DKK2* rs17037102 G/G genotype was found to be a independent favorable factor for survival after multivariate analysis (OR, 0.407; 95% CI, 0.156–1.062; p P = 0.0489) (Table 8).

Discussion

In this study, we found a significant decrease in the frequency of the G/A+A/A genotypes of the *DKK3* rs3206824 (non synonymous Arg335Gly) in RCC patients compared with controls (OR = 0.43; 95% CI, 0.29–0.65). The functions of *DKK1* and *DKK3* have been investigated and various studies have found them to be tumor suppressor genes. [12][22–32] DKK3 is a member of the Dickkopf family and regulates cell proliferation and apoptosis as a tumor suppressor gene both *in vitro* and *in vivo*.[22][23]

DKK3 mRNA and protein levels were significantly decreased in human renal cancer tissues compared to normal kidney tissues.[22] Regarding the mechanism of down-regulation of DKK3, Kobayashi et al. detected hypermethylation in the promoter region in human cancer cell lines in which the expression of DKK3 was decreased.[25]

They also detected codon335 SNP (rs3206824) in *DKK3* gene in mutation analysis and compared the distribution of genotypes between 200 healthy controls and 56 lung cancer patients. However there was no significant difference between the two groups.[25] Except for this previous report, there have been no significant findings regarding codon335 in SNP studies.

As a next step, we selected 4 polymorphic sites in the *DKK3* gene based on HapMap data, and conducted linkage disequilibrium (LD) and haplotype analysis. The *DKK3* rs3206824 (non synonymous Arg335Gly) was in linkage disequilibrium with rs7396187, and the frequency of each haplotype including rs3206824 (G/A) and rs7396187 (G/C) was calculated between RCC patients and controls. The frequency of the A–C haplotype was significantly lower in RCC ($P < 0.0001$) compared with other haplotypes. An apparent added effect was also observed in the haplotype analysis. As far as we know, this is the first report that has shown the association of the *DKK3 gene* haplotype with renal cancer.

We also selected 3 polymorphic sites in the *DKK2* gene and *DKK2* rs419558 was in linkage disequilibrium with rs447372, but there was no significant difference among the frequency of each haplotype including these two polymorphisms between RCC patients and controls. Interestingly, we found that *DKK2* rs17037102 (non synonymous Arg146Glu) G/A+A/A genotype carriers were associated with shorter survival in all patients. In addition the *DKK2* rs17037102 GG genotype was a favorable factor for survival by multivariate Cox regression analysis, suggesting that the *DKK2* rs17037102 G/A+A/A genotypes will be a useful parameter to detect high-risk RCC patients.

However, this polymorphism was not associated with other clinical variables including grade, stage, nodes or metastases. Prognostic factors are usually associated with some clinico-pathological features. However, it has been shown that functions of the Wnt signaling pathway are extremely diverse,[33] suggesting that it is currently not known whether conventional clinicopathological factors reflect all of those functions at present. This will require further study. However there has been a recent report showing that a SNP can be associated with metastasis, but not with prognosis.[34]

There are no reports regarding the *DKK2* gene polymorphism in various cancers including renal cancer, therefore we could not compare our results to other studies. Regarding DKK2 function, Kremen proteins, which are Dickkopf receptors, modulate DKK2 activity during Wnt/LRP6 signaling.[35][36] DKK2 can either activate or inhibit the Wnt/β-catenin pathway, depending on cellular context.[4] The role of *DKK2* itself on deregulation in cancer is not well understood since there are no other reports regarding *DKK2* SNPs and cancer susceptibility.

There was a marginal significance to the increased frequency of the A/A genotype of rs1802074 (*sFRP4*) in renal cancer patients compared with controls (Table 3).

We tested the gene-gene interactions among gene-gene interaction using *DKK2* rs419558, *DKK2* rs447372, *DKK3* rs3206824, *DKK3* rs7396187, *DKK4* rs2073664, *sFRP4* rs1802074, and *DAAM2* rs2504106, in which we found a significant difference in the genotype distribution between cases and controls. There was a strong correlation however between *DKK3* rs3206824 GA/AA and *sFRP4* rs1802074 AG/GG (Table 4) suggesting that these two polymorphisms are related to RCC susceptibility.

The sFRP family has been commonly reported to be down-regulated by epigenetic inactivation in various cancers.[25][37–39] Urakami et al. also investigated the methylation frequency of the *sFRP* family (*sFRP1-sFRP5*) in renal cancer tissue and adjacent normal kidney tissues and found that the methylation level of *sFRP1* was significantly higher in renal cancer tissues.[12] In other SNP studies of the *sFRP* family, Caldwell et al. found one polymorphic site on exon1 in *sFRP1*, but no significant association with the development of colorectal cancer.[38] The sFRP4 protein directly binds Wnt7a to inhibit activation of β-catenin/canonical signaling,[40][41] and was shown to be down-regulated in renal cancer

tissues by epigenetic mechanisms.[12] However at present, there are no reports investigating the potential effect of *sFRP4* gene polymorphisms on RCC.

Recently Lee et al. found that DKK3 was a negative regulator of beta-catenin.[42]

In order to investigate the effect of Wnt-antagonist polymorphisms on Wnt-signaling, we did an immunohistochemical analysis of beta-catenin expression and compared the relationship between beta-catenin expression levels and Wnt-antagonist polymorphisms. However, we did not find any relationship between beta-catenin expression and the polymorphisms. The detailed molecular mechanisms involved in how these polymorphisms have an effect on renal cancer is unclear. Nonsynonymous single nucleotide polymorphisms (nsSNPs) introduce amino acid changes and may affect protein function.[43] Therefore, it is generally believed that nsSNPs may be associated with cancer susceptibility.[43] PolyPhen (http://genetics.bwh.harvard.edu/pph/) is a computer program which is used to predict the effect of nonsynonymous coding SNPs on protein structure and function. When we used this program, *DKK3 Arg335Gly* (rs3206824), *DKK2 Arg146Glu* (rs17037102) and *sFRP4 Arg340Lys* (rs1802074) were judged to be benign, while *sFRP4 Pro320Thr* (rs1802073) showed a high PolyPhen score (1.577) and was judged as “possibly damaging”. Also *XRCC1 Arg399Gln* has been reported to be associated with the survival and prognosis of various cancer patients yet is judged to be benign by the PolyPhen program.[44][45] In addition the synonymous SNPs can alter mRNA folding and reduce mRNA stability thereby altering translation through structural changes in the RNA.[46] There is accumulating evidence regarding the functional effects of synonymous mutations. [47][48]

The polymorphisms associated with the 'Odds' of RCC are not associated with the clinical and pathological factors or survival. Similarly the polymorphisms associated with clinical and pathological features are not associated with survival. It is reasonable to consider that the functional role of a SNP as a risk factor is not always the same as that of a prognostic factor, because a risk SNP may contribute to the early stage of carcinogenesis of nearly normal cells whereas a prognostic SNP may be involved in the progression of fully transformed cells. Indeed, there have been many examples that a risk SNP is of no significance as a prognostic SNP, and vice versa.[49][50]

In conclusion, this is the first report documenting that the *DKK2* rs17037102 polymorphism may be a predictor for survival after radical nephrectomy. Although further studies with a larger sample size are necessary, our present findings contribute to an understanding of individual survival differences after nephrectomy.

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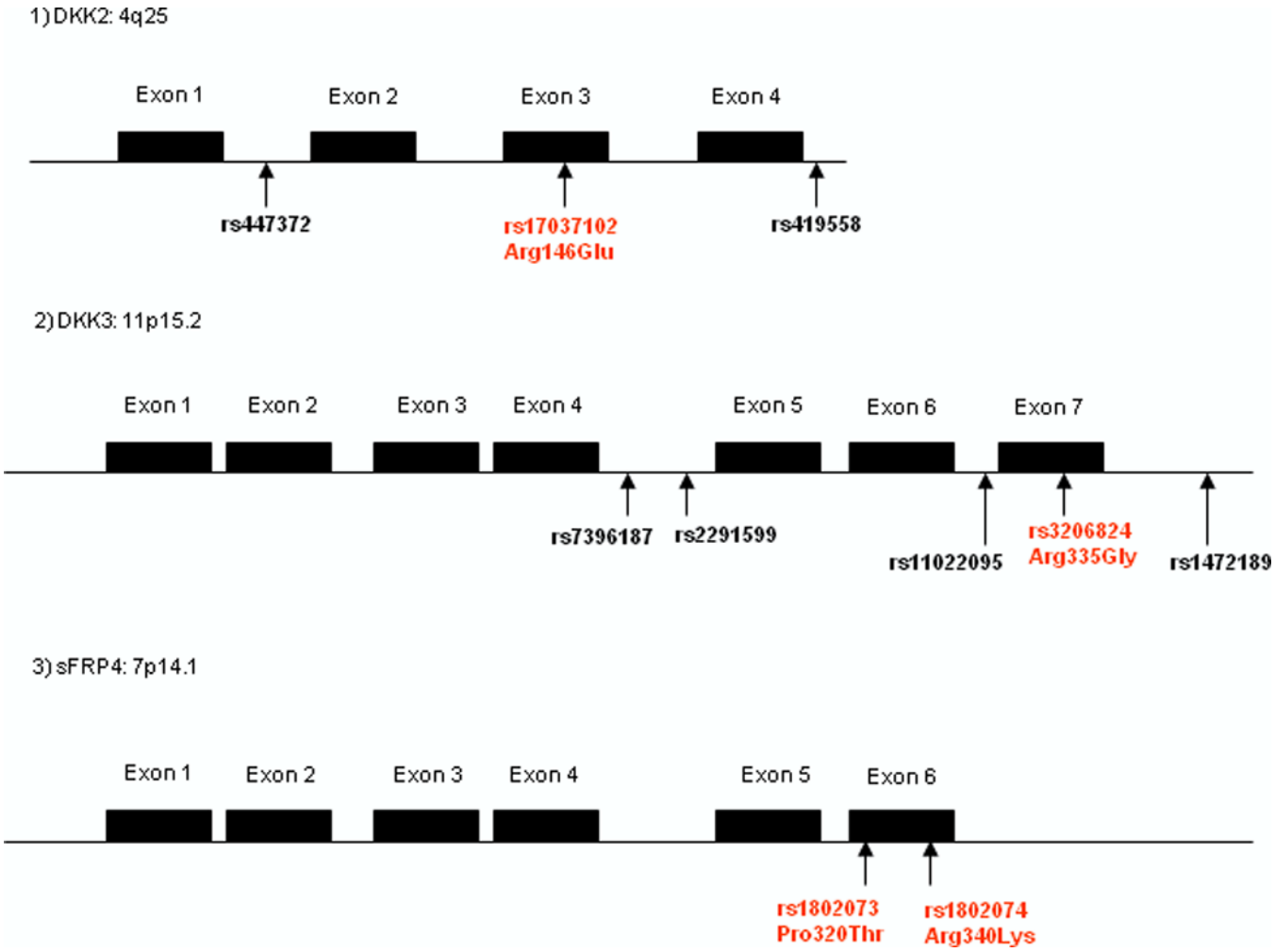

Figure 1. Diagrams of *DKK2, DKK3,* and *sFRP4* genes and polymorphic sites
1) DKK2 2) DKK3 3) sFRP4

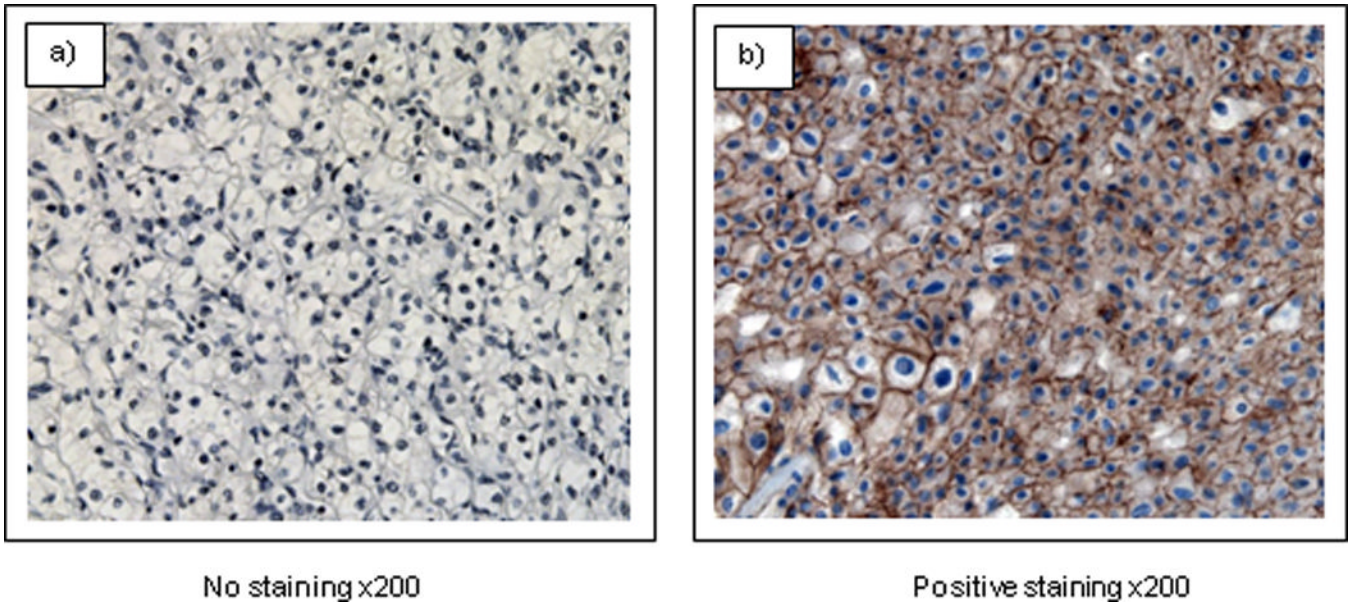

Figure 2. Representative immunohistochemical stains of beta-catenin in RCC tissues
RCC cells showed mainly cytoplasmic and membranous expression.
Magnification is ×200

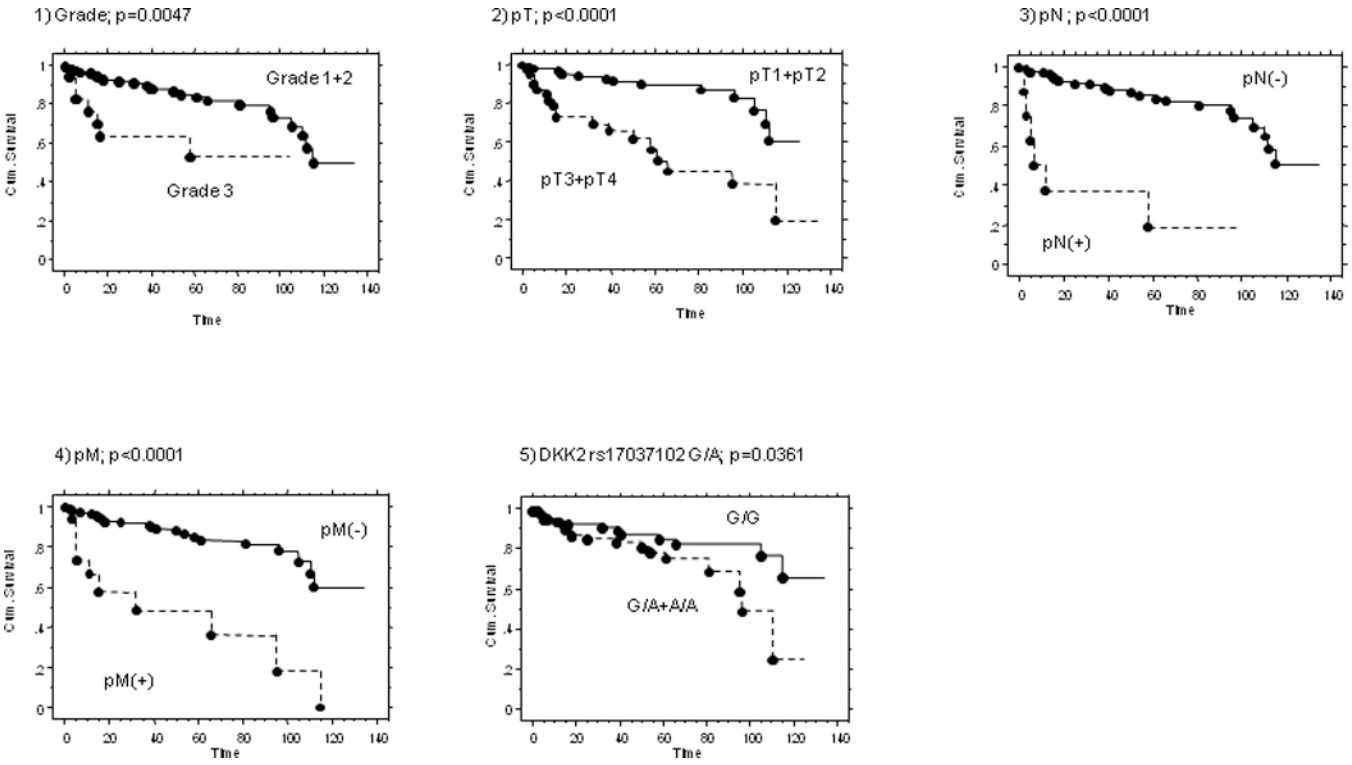

Figure 3. Kaplan-Meier survival curve for overall survival for 160 RCC patients

(1) grade (2) pT (3) pN (4) pM (5) *DKK2* rs17037102

Higher grade (grade 3 + 4), pT3 + pT4, pN(+), pM(+), and *DKK2* rs17037102 G/A+A/A genotypes are independent predictors of overall survival.

Table 1

Characteristics of RCC patients and controls

		Cases (n=210)	Controls (n=200)	P-value
Age(y)(mean ± SD)		62.1 ± 12	60.1 ± 16	0.35
Gender	Males	145(69 %)	151 (75%)	0.23
	Females	65 (31 %)	49 (25 %)	
Grade	1	55 (26 %)		
	2	130 (62 %)		
	3+4	25(12 %)		
pT	1	114 (54 %)		
	2	45(21 %)		
	3	48 (23%)		
	4	3(2%)		
pN	(−)	200 (95 %)		
	(+)	10 (5%)		
pM	(−)	191 (91 %)		
	(+)	19(9 %)		
Pathology				
	Clear cell carcinoma	198 (94 %)		
	Granular cell carcinoma	10 (5 %)		
	Chromophobe cell carcinoma	2(1 %)		

Abbreviations: pT, pathological tumor classification; pN, lymph node invasion; pM, distant metastasis

Table 2

Information about SNP ID, function variation, primers sequence, product size, PCR conditions, and restriction enzymes for the target genes

gene	SNP ID	function variation			primer sequence	product size	annealing Tem (cycle number)	restriction enzyme
DKK2	rs17037102	non synonymous	1 st	F;	5'-TGGCTTCATATTTCACATCAAGA-3'		52 (48)	
		exon3 Arg146Glu		R;	5'-TGTGTGGTCTTCCTAGATTCTGC-3'			
			2 nd	F;	5'-TGATCATCTCCAGGCATCTG-3'	132bp	58 (40)	***DdeI***
				R;	5'-ATTCTGCCATCCCAAGTCAT-3'			
DKK2	rs419558	3'UTR	1 st	F;	5'-TACGGACACAGGACCTCACA-3'		52 (48)	
				R;	5'-TCCAATATATGTGGGAAAAGAGC-3'			
			2 nd	F;	5'-TCCCCTGGTTTCAAAGATGA-3'	251bp	58 (40)	***AflII***
				R;	5'-TGGGAAAAGAGCTAACAGAGAGA-3'			
DKK2	rs447372	intron1	1 st	F;	5'-TGCTCAAAAAGCAGCATCTC-3'		52 (48)	
				R;	5'-AGCTAACGGTCACTCAACCAA-3'			
			2 nd	F;	5'-TCAAAAAGCAGCATCTCAGG-3'	249bp	58 (40)	***EcoRV***
				R;	5'-CTCCAAATTTTGCCCTACCA-3'			
DKK3	rs3206824	non synonymous	1 st	F;	5'-GAGGTCCCCGATGAGTATGA-3'		52 (48)	
		exon7 Arg335Gly		R;	5'-TAGGAAGAAGCCTGGTCAGC-3'			
			2 nd	F;	5'-GGTCCCCGATGAGTATGAAG-3'	209bp	58 (40)	***DdeI***
				R;	5'-AGCACACACCTGGGGAAATA-3'			
DKK3	rs11022095	intron 6 A/G	1 st	F;	5'-TCAAGCAATCCTCCTGCATT-3'		52 (48)	
				R;	5'-AGTGGATGGTCCATGGAGAG-3'			
			2 nd	F;	5'-TTACAGGTGTGAGCCACTGC-3'	288bp	58 (40)	***BssSI***
				R;	5'-CTCCCCAGACCTGTCACAAT-3'			
DKK3	rs1472189	3' near gene C/T	1 st	F;	5'-TGGGGGACCTAGTTTTATCTCA-3'		52 (48)	
				R;	5'-ACCATCAGGACCACCCTACA-3'			
			2 nd	F;	5'-CTTCCTCAAGCTTTCCTTGC-3'		58 (40)	***BslI***
				R;	5'-TACCTTGAAGGCATCCCAGT-3'			
DKK3	rs7396187	intron4 G/C	1 st	F;	5'-TTCCTTAGGTCCCTAGGTCCA-3'		52 (48)	
				R;	5'-AGGGCAAAGGAGACTCTTCA-3'			
			2 nd	F;	5'-ACAGGGCATGGCAGTTAGAG-3'	245bp	58 (40)	***Fnu4HI***
				R;	5'-CTCTTCACCCAACAGGCATT-3'			
DKK3	rs2291599	intron4 C/T	1 st	F;	5'-CAGAGGACATGGGGTGGAT-3'		52 (48)	

gene	SNP ID	function variation			primer sequence	product size	annealing Tem (cycle number)	restriction enzyme
				R;	5'-CTGCTGCTCTGCTCTCCATT-3'			
			2 nd	F;	5'-CTTGTCCTCCAGGAGTCAGC-3'	250bp	58 (40)	***AvaI***
				R;	5'-CATCATCGACGAGGACTGTG-3'			
DKK4	rs2073664	synonymous	1 st	F;	5'-GCCATGGCATTACTGCTTTT-3'		52 (48)	
		exon 4 V169V		R;	5'-ATTGCTGGTCAATTGGCTTC-3'			
			2 nd	F;	5'-CTGCGTGCTGTGTCTGTTTT-3'	292bp	58 (40)	***EcoNI***
				R;	5'-AACGCTGGAAGATTTCTGGA-3'			
SFRP4	rs1802073	non synonymous	1 st	F;	5'-GAGCACCATAAAGGGGTGAG-3'		52 (48)	
		exon6 P320T		R;	5'-GGGCACATGGCCTTACATAG-3'			
			2 nd	F;	5'-ACAGCGGAGAACAGTTCAGG-3'	246bp	58 (40)	***HinfI***
				R;	5'-TGGCCTTACATAGGCTGTCC-3'			
SFRP4	rs1802074	non synonymous	1 st	F;	5'-AAGAGAGGCTGCAGGAACAG-3'		52 (48)	
		exon6 R340K		R;	5'-TCTGTACCAAAGGGCAAACC-3'			
			2 nd	F;	5'-AGAGCGGAGAACAGTTCAGG-3'	246bp	58 (40)	***EarI***
				R;	5'-TGGCCTTACATAGGCTGTCC-3'			
SMAD7	rs12953717	intron3 C/T	1 st	F;	5'-GTGCCACAGGGTCTCCTTC-3'		52 (48)	
				R;	5'-GGATGTGGAGACAATCAGGAA-3'			
			2 nd	F;	5'-GCTTCGTTTCCACCCCTTAG-3'	272bp	58 (40)	***Fnu4HI***
				R;	5'-AACCCAGGAGCCTCAGAGAT-3'			
DAAM2	rs6937133	intron1 A/G	1 st	F;	5'-GCACTGGTGCTCACTCCTCT-3'		52 (48)	
				R;	5'-TTCCTGCACAGCTGAGTGTC-3'			
			2 nd	F;	5'-TTGCAGTTAAACCTGGGTGA-3'	175bp	58 (40)	***TaqI***
				R;	5'-TGTTCGCTGCCCAGTTTAGT-3'			
DAAM2	rs2504106	intron1 C/T	1 st	F;	5'-TGGCCTCTCATACATCATGC-3'		52 (48)	
				R;	5'-CCCTCTTTCCTCCTTTTCCA-3'			
			2 nd	F;	5'-GCCCAGAAAAGCCTCAAATA-3'	215bp	58 (40)	***SfcI***
				R;	5'-GGTCATTTCCCAAATGGTCA-3'			

Table 3

Association between polymorphisms in Wnt signaling pathway genes and renal cancer

Gene polymorphism	Genotype	renal cancer (n=210)	control (n=200)	OR (95% CI)	p-value	FDR adjusted p-value*
		n (%)	n (%)			
DKK2 (rs17037102)	GG	105 (50)	106 (53)	1 (reference)		
	GA	92 (44)	82 (41)	1.13 (0.76–1.69)	0.54	
	AA	12 (6)	12 (06)	1.01 (0.43–2.34)	0.98	
	GA+AA	104 (50)	94 (47)	1.11 (0.76–1.65)	0.57	0.61
DKK2 (rs419558)	CC	119 (57)	88 (44)	1 (reference)		
	CT	63 (30)	88 (44)	0.53 (0.34–0.81)	0.003	
	TT	28 (13)	24 (12)	0.86 (0.45–1.58)	0.64	
	CT+TT	91 (43)	112 (56)	0.60 (0.41–0.88)	0.01	**0.028**[a]
DKK2 (rs447372)	GG	117 (56)	88 (44)	1 (reference)		
	GA	65 (31)	88 (44)	0.56 (0.36–0.84)	0.0063	
	AA	28 (13)	24 (12)	0.88 (0.47–1.62)	0.67	
	GA+AA	93 (44)	112 (56)	0.62 (0.42–0.92)	0.01	**0.0238**[a]
DKK3 (rs3206824)	GG	150 (71)	104 (52)	1 (reference)		
	GA	53 (25)	82 (41)	0.45 (0.29–0.69)	0.0001	
	AA	7 (4)	14 (7)	0.35 (0.14–0.89)	0.02	
	GA+AA	60 (29)	96 (48)	0.43 (0.29–0.65)	0.0001	**0.0014**[a]
DKK3 (rs11022095)	GG	28 (13)	23 (11)	1 (reference)		
	GA	88 (42)	87 (44)	0.83 (0.44–1.55)	0.56	
	AA	94 (45)	90 (45)	0.85 (0.46–1.59)	0.63	
	GA+AA	182 (87)	177 (89)	0.84 (0.47–1.52)	0.57	0.57
DKK3 (rs1472189)	CC	155 (74)	134 (67)	1 (reference)		
	CT	51 (24)	59 (30)	0.74 (0.48–1.16)	0.19	
	TT	4 (2)	7 (3)	0.49 (0.14–1.72)	0.26	
	CT+TT	55 (26)	66 (33)	0.72 (0.47–1.10)	0.13	0.18
DKK3 (rs7396187)	GG	138 (66)	102 (51)	1 (reference)		
	GC	58 (27)	75 (38)	0.57 (0.37–0.88)	0.01	

Gene polymorphism	Genotype	renal cancer (n=210)	control (n=200)	OR (95% CI)	p-value	FDR adjusted p-value*
		n (%)	n (%)			
	CC	14 (7)	23 (11)	0.45 (0.22–0.92)	0.02	
	GC+CC	72 (34)	98 (49)	0.54 (0.37–0.81)	0.002	**0.01**[a]
DKK3 (rs2291599)	CC	101 (48)	113 (57)	1 (reference)		
	CT	81 (39)	62 (31)	1.46 (0.95–2.24)	0.08	
	TT	28 (13)	25 (12)	1.25 (0.69–2.29)	0.46	
	CT+TT	109 (52)	87 (43)	1.41 (0.95–2.07)	0.08	0.14
DKK4 (rs2073664)	CC	144 (68)	145 (72)	1 (reference)		
	CT	40 (19)	46 (23)	0.88 (0.54–1.42)	0.58	
	TT	26 (13)	9 (5)	2.91 (1.32–6.42)	0.006	
	CT+TT	66 (32)	55 (28)	1.21 (0.79–1.85)	0.38	0.48
sFRP4 (rs1802073)	CC	51 (24)	36 (18)	1 (reference)		
	CA	82 (39)	112 (56)	0.52 (0.31–0.86)	0.01	
	AA	77 (37)	52 (26)	1.04 (0.61–1.82)	0.87	
	CA+A	159 (76)	164 (82)	0.68 (0.42–1.11)	0.12	0.19
sFRP4 (rs1802074)	AA	36 (17)	18 (9)	1 (reference)		
	AG	69 (33)	69 (34)	0.51 (0.26–0.96)	0.04	
	GG	105 (50)	113 (56)	0.46 (0.25–0.87)	0.01	
	AG+GG	174 (83)	182 (91)	0.48 (0.26–0.87)	0.01	**0.04**[a]
SMAD7 (rs12953717)	CC	125 (59)	139 (70)	1 (reference)		
	CT	68 (32)	48 (24)	1.57 (1.01–2.45)	0.04	
	TT	17 (9)	13 (6)	1.45 (0.68–3.11)	0.33	
	CT+TT	85 (41)	61 (30)	1.55 (1.03–2.33)	0.03	0.06
DAAM2 (rs6937133)	GG	30 (14)	33 (16)	1 (reference)		
	GA	65 (31)	65 (33)	1.1(0.61–2.01)	0.75	
	AA	115 (55)	102 (51)	1.24 (0.71–2.17)	0.45	
	GA+AA	180 (86)	167 (84)	1.18 (0.69–2.03)	0.53	0.62
DAAM2 (rs2504106)	TT	72 (34)	37 (17)	1 (reference)		
	TC	84 (40)	118 (59)	0.36 (0.23–0.59)	0.0001	
	CC	54 (26)	45 (23)	0.62 (0.35–1.08)	0.08	

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Gene polymorphism	Genotype	renal cancer (n=210)	control (n=200)	OR (95% CI)	p-value	FDR adjusted p-value*
		n (%)	n (%)			
	TC+CC	138 (66)	163 (82)	0.44 (0.28–0.69)	0.0002	**0.0014**[a]

Note; FDR means false discovery rate, FDR adjusted P value using the Benjamini-Hochberg methods.

FDR adjusted p-value of < 0.05 was regarded as statistically significant,

[a]Remain significant after FDR adjustment

Table 4

Gene-gene interaction analysis in *DKK3* rs3206824-*sFRP4* rs1802074

***DKK3* A (GG/GA+AA)-*sFRP4* B (AA/AG+GG)**	**renal cancer (n=210) n (%)**	**control (n=200) n (%)**	**OR (95% CI)**	**p-value**
GG-AA	27 (13)	9 (4.5)	1 (reference)	
GG-AG/GG	123 (58)	95 (47.5)	0.43 (0.19–0.96)	0.04
GA/AA-AA	9 (5)	9 (5)	0.33 (0.10–0.20)	0.06
GA/AA-AG/GG	51 (24)	87 (43.5)	0.19 (0.09–0.45)	<0.0001

Table 5

Haplotype analysis and renal cancer risk
A) DKK3 rs3206824-rs7396187
B) DKK2 rs419558-rs447372

A)

Haplotype DKK3 A (G/A)-F (G/C)	Overall	case (%)	cont (%)	p-value
G-G	0.719	0.7661	0.6701	<0.001
A-C	0.1873	0.1321	0.2447	<0.001
G-C	0.0651	0.0736	0.0563	0.3155
A-G	0.0285	0.0281	0.0289	0.9451

B)

Haplotype DKK2 A (C/T)- B (G/A)	Overall	case (%)	control (%)	p-value
C-G	0.6866	0.7119	0.66	0.109
T-A	0.311	0.2833	0.34	0.0796
C-A	2.44E-03	4.76E-03	5.10E-28	0.167

Note; A: rs3206824, F: rs7396187

Note; A: rs419558, B: rs447372

Table 6

Comparison between gene genotypes and clinical parameters

Gene (SNP ID)	Genotype	gender			Grade			Stage			pN			pM		
		female	male	p value	1+2	3+4	p value	pT1+pT2	pT3+pT4	p value	(−)	(+)	p value	(−)	(+)	p value
		n=65	n=145		n=185	n=25		n=159	n=51		n=200	n=10		n=191	n=19	
DKK2 (rs17037102)	GG	35	70	reference	92	13	reference	79	26	reference	99	6	reference	94	11	reference
	GA	24	68	0.27	82	10	0.74	70	22	0.89	89	3	0.41	85	7	0.49
	AA	6	7	0.36	11	2	0.76	10	3	0.89	12	1	0.78	12	1	0.75
	GA+AA	30	75	0.45	93	12	0.83	80	25	0.87	101	4	0.52	97	8	0.47
DKK2 (rs419558)	CC	39	80	reference	107	12	reference	91	28	reference	118	1	reference	113	6	reference
	CT	17	46	0.42	56	7	0.83	48	15	0.96	57	6	0.004	54	9	0.03
	TT	9	19	0.94	22	6	0.11	20	8	0.58	25	3	0.004	24	4	0.08
	CT+TT	26	65	0.51	78	13	0.35	68	23	0.77	82	9	0.002	78	13	0.02
DKK2 (rs447372)	GG	39	78	reference	105	12	reference	90	27	reference	116	1	reference	111	6	reference
	GA	17	48	0.31	58	7	0.91	49	16	0.82	59	6	0.005	56	9	0.04
	AA	9	19	0.91	22	6	0.11	20	8	0.54	25	3	0.004	24	4	0.09
	GA+AA	26	67	0.41	80	13	0.41	69	24	0.65	84	9	0.003	80	13	0.03
DKK3 (rs3206824)	GG	50	100	reference	136	14	reference	116	34	reference	143	7	reference	138	12	reference
	GA	13	40	0.23	43	10	0.06	37	16	0.28	50	3	0.77	46	7	0.26
	AA	2	5	0.79	6	1	0.66	6	1	0.61	7	0		7	0	
	GA+AA	15	45	0.24	49	11	0.07	43	17	0.39	57	3	0.92	53	7	0.41
DKK3 (rs11022095)	GG	11	17	reference	25	3	reference	21	7	reference	27	1	reference	23	5	reference
	GA	28	60	0.47	76	12	0.68	67	21	0.91	83	5	0.61	80	8	0.21
	AA	26	68	0.24	84	10	0.99	71	23	0.95	90	4	0.87	88	6	0.06
	GA+AA	54	128	0.31	160	22	0.83	138	44	0.93	173	9	0.75	168	14	0.08
DKK3 (rs1472189)	CC	47	108	reference	138	17	reference	119	36	reference	150	5	reference	146	9	reference
	CT	16	35	0.89	46	5	0.82	38	13	0.74	46	5	0.05	41	10	**0.003**
	TT	2	2	0.41	1	3	**0.0001**	2	2	0.21	4	0		4	0	
	CT+TT	18	37	0.74	47	8	0.48	40	15	0.55	50	5	0.07	45	10	**0.005**
DKK3 (rs7396187)	GG	46	92	reference	127	11	reference	108	30	reference	132	6	reference	127	11	reference
	GC	16	42	0.43	46	12	**0.01**	40	18	0.11	54	4	0.46	51	7	0.36
	CC	3	11	0.36	12	2	0.42	11	3	0.92	14	0		13	1	0.92

Gene (SNP ID)	Genotype	gender			Grade			Stage			pN			pM		
		female	male	p value	1+2	3+4	p value	pT1+pT2	pT3+pT4	p value	(−)	(+)	p value	(−)	(+)	p value
		n=65	n=145		n=185	n=25		n=159	n=51		n=200	n=10		n=191	n=19	
	GC+CC	19	53	0.31	58	14	**0.014**	51	21	0.14	68	4	0.69	64	8	0.45
DKK3 (rs2291599)	CC	34	67	reference	93	8	reference	76	25	reference	95	6	reference	90	11	reference
	CT	26	55	0.82	70	11	0.21	62	19	0.84	77	4	0.77	74	7	0.61
	TT	5	23	0.11	22	6	**0.04**	21	7	0.98	28	0		27	1	0.24
	CT+TT	31	78	0.41	92	17	0.08	83	26	0.88	105	4	0.44	101	8	0.37
DKK4 (rs2073664)	CC	40	104	reference	127	17	reference	107	37	reference	137	7	reference	129	15	reference
	CT	18	22	**0.03**	35	5	0.91	30	10	0.92	37	3	0.52	36	4	0.94
	TT	7	19	0.93	23	3	0.96	22	4	0.26	26	0		26	0	
	CT+TT	25	41	0.14	58	8	0.95	52	14	0.48	63	3	0.92	62	4	0.31
sFRP4 (rs1802073)	CC	15	36	reference	42	9	reference	37	14	reference	47	4	reference	43	7	reference
	CA	25	57	0.89	70	12	0.64	65	17	0.37	79	3	0.29	76	6	0.21
	AA	25	52	0.71	73	4	**0.02**	57	20	0.85	74	3	0.34	71	6	0.26
	CA+AA	50	109	0.78	143	16	0.15	122	37	0.54	153	6	0.24	147	12	0.17
sFRP4 (rs1802074)	AA	15	21	reference	32	4	reference	28	8	reference	35	1	reference	35	1	reference
	AG	15	54	**0.03**	65	4	0.33	53	16	0.92	67	2	0.97	64	5	0.35
	GG	35	70	0.36	88	17	0.09	78	27	0.68	98	7	0.38	92	13	0.09
	AG+GG	50	124	0.13	153	21	0.87	131	43	0.75	165	9	0.54	156	18	0.15
SMAD7 (rs12953717)	CC	46	79	reference	108	17	reference	94	31	reference	119	6	reference	111	14	reference
	CT	13	55	**0.01**	61	7	0.51	51	17	0.98	65	3	0.91	65	3	0.11
	TT	6	11	0.91	16	1	0.37	14	3	0.52	16	1	0.85	15	2	0.95
	CT+TT	19	66	**0.03**	77	8	0.36	65	20	0.83	81	4	0.97	80	5	0.19
DAAM2 (rs6937133)	GG	12	18	reference	26	4	reference	22	8	reference	29	1	reference	26	4	reference
	GA	20	45	0.37	59	6	0.54	48	17	0.96	62	3	0.77	56	9	0.95
	AA	33	82	0.23	100	15	0.97	89	26	0.64	109	6	0.67	109	6	0.12
	GA+AA	53	127	0.25	159	21	0.79	137	43	0.74	171	9	0.69	165	15	0.38
DAAM2 (rs2504106)	TT	19	53	reference	64	8	reference	57	15	reference	68	4	reference	66	6	reference
	TC	29	55	0.27	73	11	0.71	62	22	0.43	80	4	0.83	73	11	0.34
	CC	17	37	0.53	48	6	0.99	40	14	0.51	52	2	0.63	52	2	0.29
	TC+CC	46	92	0.31	121	17	0.79	102	36	0.39	132	6	0.69	125	13	0.79

note; Considering multiple comparison,

[a] p-value of < 0.05 was regarded as statistically significant

Table 7

Relationship of Wnt antagonist genes distribution with the expression of beta-catenin

		beta catenin expression (IHC)		
		NO (n=25)	YES (n=31)	p value
Gene polymorphism	Genotype	n (%)	n (%)	
DKK2 (rs17037102)	GG	18(72)	21(68)	reference
	GA	5(20)	9(29)	0.49
	AA	2(8)	1(3)	0.49
	GA+AA	7(28)	10(32)	0.73
DKK2 (rs419558)	CC	16(64)	22(71)	reference
	CT	6(24)	3(10)	0.18
	TT	3(12)	6(19)	0.63
	CT+TT	9(36)	9(29)	0.58
DKK2 (rs447372)	GG	16(64)	22(71)	reference
	GA	7(28)	3(10)	0.12
	AA	2(8)	6(19)	0.37
	GA+AA	9(36)	9(29)	0.58
DKK3 (rs3206824)	GG	21(84)	24(77)	reference
	GA	4(16)	6(19)	0.72
	AA	0(0)	1(4)	
	GA+AA	4(16)	7(23)	0.54
DKK3 (rs11022095)	GG	5(20)	4(13)	reference
	GA	7(35)	10(32)	0.48
	AA	13(45)	17(55)	0.52
	GA+AA	20(80)	27(87)	0.47
DKK3 (rs1472189)	CC	23(92)	23(74)	reference
	CT	2(8)	8(26)	0.08
	TT	0(0)	0	
	CT+TT	2(8)	8(26)	0.08
DKK3 (rs7396187)	GG	21(84)	23(74)	reference
	GC	4(16)	5(16)	0.85
	CC	0(0)	3(10)	
	GC+CC	4(16)	8(26)	0.38
DKK3 (rs2291599)	CC	11(44)	15(48)	reference
	CT	9(36)	11(35)	0.86
	TT	5(20)	5(16)	0.67
	CT+TT	14(56)	16(52)	0.74
DKK4 (rs2073664)	CC	16(64)	21(68)	reference
	CT	6(24)	3(10)	0.21
	TT	3(12)	7(22)	0.45
	CT+TT	9(36)	10(32)	0.77
sFRP4 (rs1802073)	CC	6(24)	10(32)	reference

		beta catenin expression (IHC)		
		NO (n=25)	YES (n=31)	p value
Gene polymorphism	Genotype	n (%)	n (%)	
	CA	8(32)	11(35)	0.78
	AA	11(44)	10(32)	0.37
	CA+AA	19(76)	21(68)	0.49
sFRP4 (rs1802074)	AA	8(32)	9(29)	reference
	AG	12(48)	11(35)	0.75
	GG	5(20)	11(35)	0.35
	AG+GG	17(68)	22(71)	0.82

Table 8

Multivariate Cox proportional hazards analysis for overall survival after radical nephrectomy (n=160)

parameters	Hazard Ratio (95% CI)	p value
grade; 3 vs 1+2	1.515 (0.411–5.584)	0.5324
pT; pT3+4 vs pT1+pT2	4.017 (1.469–10.986)	0.0067
pN; pN(+) vs pN(−)	8.885 (1.368–57.713)	0.0221
pM; pM(+) vs pM(−)	7.312 (2.003–26.692)	0.0026
DKK2 rs17037102 G/G vs G/A+A/A	0.407 (0.156–1.062)	0.0489