Probes for the *ERC2* gene were designed for the Infinium array, and DNA hypermethylation around the 5′-region of the *ERC2* gene was detected in only 6% of RCCs, indicating that reduced expression of the *ERC2* gene may not be attributable to DNA methylation alterations during renal carcinogenesis. Since the probes for the *ABCA13* gene were not designed for the Infinium array, we examined DNA methylation levels in the 5′-region of the *ABCA13* gene by pyrosequencing. No significant differences in the DNA methylation levels of the *ABCA13* gene between T samples (0.528 ± 0.060, $n = 67$) and N samples (0.510 ± 0.149, $n = 67$) were observed (Supporting Information Fig. S2a). Our data for RCCs were consistent with the data in the public database Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/): no significant differences in DNA methylation levels of the *ABCA13* gene were evident between bile duct cancer and normal bile duct tissue (Accession number: GSE49656) and between breast cancer and normal breast tissue (GSE37754), indicating that reduced expression of the *ABCA13* gene may not be attributable to DNA methylation alterations during renal carcinogenesis.

Alterations of expression associated with DNA hypermethylation or hypomethylation

All genes showing DNA methylation alterations [0.2 or more $\Delta\beta$ ($\beta_T - \beta_N$) or -0.2 or less $\Delta\beta$ ($\beta_T - \beta_N$)] or mRNA expression alterations [4 or more ΔE ($E_T - E_N$) or -4 or less ΔE ($E_T - E_N$)] in each RCC are summarized in Supporting Information Table S6 along with genes showing genetic aberration scores of 1 or more. The DNA methylation status of the 5′-region can regulate the mRNA expression level of each gene. DNA methylation status is stably preserved on DNA double strands by covalent bonds and inherited through cell division by maintenance-methylation mechanisms by *DNMT1*. Therefore, altered mRNA expression due to DNA methylation alterations may be more stably fixed during multistage human carcinogenesis in comparison to mRNA expression alterations without DNA methylation alterations. Therefore, we have calculated upregulation and downregulation scores based on both DNA methylation status and expression levels described in the Material and Methods section: 86 genes showed reduced expression [-4 or less ΔE ($E_T - E_N$)] associated with DNA hypermethylation [0.2 or more $\Delta\beta$ ($\beta_T - \beta_N$)] in 5 or more patients (downregulation scores of 5 or more; Table 2) and 28 genes showed overexpression [4 or more ΔE ($E_T - E_N$)] associated with DNA hypomethylation [-0.2 or less $\Delta\beta$ ($\beta_T - \beta_N$)] in 5 or more patients (upregulation scores of 5 or more; Table 2).

Expression alterations of genes included in Table 2 were validated using the clear cell RCC database in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; Supporting Information Table S7): reduced or increased mRNA expression of 97 (89%) of the109 genes, which are included in Table 2 and for which probes were designed in the expression microarrays described in the database, were found, indicating the reliability of our expression analysis. Since genome-wide DNA methylation data for RCCs obtained using array-based analysis with appropriate resolution were not available in the public database, Infinium assay data for other human malignant tumors deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) were used instead for validation (Supporting Information Table S8). In addition, DNA methylation levels of the representative genes, *RAB25, GGT6, C3* and *CHI3L2*, included in Table 2 based on the Infinium assay were successfully verified using pyrosequencing (Supporting Information Figs. S2*b*–S2*e*), indicating the reliability of our Infinium assay.

Pathway analysis

MetaCore pathway analysis by GeneGo was performed for 61 genes assigned genetic aberration scores of 3 or more, 86 genes assigned downregulation scores of 5 or more (frequent reduction of expression associated with DNA hypermethylation) and 28 genes assigned upregulation scores of 5 or more (frequent overexpression associated with DNA hypomethylation; total 174 genes). Twenty potentially significant GeneGo pathways ($p < 0.05$) and the affected genes are listed in Table 3. Mutations of 5 (100%) of the 5 genes included in Table 3 were found in the clear cell RCC database of The Cancer Genome Atlas (Supporting Information Table S5). Reduced or increased mRNA expression of 11 (92%) of the 12 genes, which are included in Table 3 and for which probes had been designed in expression microarrays described in the clear cell RCC database of the Gene Expression Omnibus, were found (Supporting Information Table S7), supporting the participation of these genes in renal carcinogenesis.

Genes for which correlation with Wnt/β-catenin signaling was indicated by MetaCore pathway analysis, together with their genetic aberration, DNA methylation alterations and mRNA expression alterations, are illustrated schematically in Figure 1. Mutations, mRNA expression alterations or DNA methylation alterations of 32 (89%) of the 36 genes included in Figure 1 were found in Supporting Information Tables S5, S7 or S8, supporting the participation of the Wnt/β-catenin signaling pathway in renal carcinogenesis. In addition, MetaCore pathway analysis was separately performed for RCCs with and without genetic aberrations and/or DNA hypermethylation [$\Delta\beta$ ($\beta_T - \beta_N$) >0.2] of the *VHL* gene (Supporting Information Table S9 and Fig. S3).

Discussion

High frequencies of genetic aberrations of the *VHL* (53%), *PBRM1* (33%), *KDM5C* (12%) and *SETD2* (9%) genes, which have been highlighted in previous resequencing[2] and exome analyses,[4,6] supported the reliability of our approach. In addition to *PBRM1*, somatic mutation of another member of the SWI/SNF complex, *SMARCA4*, was detected. In addition to *SETD2* and *KDM5C*, somatic mutation of another histone modification protein, *JARID2*, was also detected. The significance of aberrations of chromatin remodeling and histone modification-related proteins in RCCs was confirmed.

Table 2. Genes showing downregulation or upregulation scores of 5 or more in clear cell RCCs

Gene	Chromosome	Entrez GeneID	Downregulation score[1]
(a) Genes showing reduced mRNA expression associated with DNA hypemethylation in their 5′-regions			
CLCNKB	1	1,188	24
SCNN1A	12	6,337	24
RAB25	1	57,111	22
TMEM213	7	155,006	22
ATP6V0A4	7	50,617	22
NR0B2	1	8,431	21
KCNJ1	11	3,758	21
GGT6	17	124,975	21
CLDN8	21	9,073	20
CLDN19	1	149,461	19
MUC15	11	143,662	16
RANBP3L	5	202,151	15
HRG	3	3,273	14
TSPAN8	12	7,103	14
RGS7	1	6,000	11
PTH1R	3	5,745	11
CWH43	4	80,157	11
F11	4	2,160	11
IRX2	5	153,572	11
EHF	11	26,298	11
CBLC	19	23,624	11
ATP6V1B1	2	525	10
LRRC2	3	79,442	10
CLDN16	3	10,686	10
EGF	4	1,950	10
WISP3	6	8,838	10
PHYHD1	9	254,295	10
FLJ45983	10	399,717	10
WIT-AS	11	51,352	10
ACSF2	17	80,221	10
ALDOB	9	229	9
ANKRD2	10	26,287	9
WT1	11	7,490	9
SOST	17	50,964	9
CYP4F3	19	4,051	9
COL18A1-AS1	21	378,832	9
BSND	1	7,809	8
TACSTD2	1	4,070	8
SLC44A4	6	80,736	8
KHDRBS2	6	202,559	8
VWC2	7	375,567	8

Table 2. Genes showing downregulation or upregulation scores of 5 or more in clear cell RCCs (Continued)

Gene	Chromosome	Entrez GeneID	Downregulation score[1]
CHRM1	11	1,128	8
COL4A6	X	1,288	8
XPNPEP2	X	7,512	8
PROM2	2	150,696	7
ACPP	3	55	7
CKMT2	5	1,160	7
NEFM	8	4,741	7
KCNA4	11	3,739	7
FLRT1	11	23,769	7
OLFM4	13	10,562	7
SERPINA4	14	5,267	7
STRA6	15	64,220	7
CRABP1	15	1,381	7
SLC7A10	19	56,301	7
CSDC2	22	27,254	7
VWA5B1	1	127,731	6
LAD1	1	3,898	6
SYN2	3	6,854	6
SLC22A13	3	9,390	6
ABHD14A	3	25,864	6
UPK1B	3	7,348	6
KCTD8	4	386,617	6
SFRP1	8	6,422	6
GATA3	10	2,625	6
DAO	12	1,610	6
TMPRSS3	21	64,699	6
CHD5	1	26,038	5
PRELP	1	5,549	5
PLD5	1	200,150	5
MAL	2	4,118	5
ENTPD3	3	956	5
TNNC1	3	7,134	5
ANK2	4	287	5
PART1	5	25,859	5
SVOPL	7	136,306	5
DMRT2	9	10,655	5
AMBP	9	259	5
RBP4	10	5,950	5
SLC22A12	11	116,085	5
PDZRN4	12	29,951	5
PROZ	13	8,858	5
RHCG	15	51,458	5
KLK6	19	5,653	5

Table 2. Genes showing downregulation or upregulation scores of 5 or more in clear cell RCCs (Continued)

Gene	Chromosome	Entrez GeneID	Downregulation score[1]
BEX1	X	55,859	5
ZCCHC16	X	340,595	5
Gene	**Chromosome**	**Entrez GeneID**	**Up-regulation score[2]**
(b) Genes showing increased mRNA expression associated with DNA hypomethylation in their 5′-regions.			
CA9	9	768	25
C3	19	718	23
CP	3	1,356	22
NNMT	11	4,837	21
FABP7	6	2,173	11
REG1A	2	5,967	10
UBD	6	10,537	8
ENPP3	6	5,169	8
MCHR1	22	2,847	7
FCGR3A	1	2,214	6
FGG	4	2,266	6
PMCHL1	5	5,369	6
CPA6	8	57,094	6
SAA2	11	6,289	6
SAA1	11	6,288	6
DNAJB13	11	374,407	6
VWF	12	7,450	6
FGF11	17	2,256	6
SPAG4	20	6,676	6
CHI3L2	1	1,117	5
FCRL3	1	115,352	5
TIGIT	3	201,633	5
APOLD1	12	81,575	5
CCL18	17	6,362	5
CARD14	17	79,092	5
LILRA2	19	11,027	5
CXorf36	X	79,742	5
SH2D1A	X	4,068	5

[1]If the probe of the Infinium array was designed in the 5′-region of the gene, if $\Delta\beta$ ($\beta_T - \beta_N$) was 0.2 or more (DNA hypermethylation) and if ΔE ($E_T - E_N$) based on the expression microarray was −4 or less (reduced expression) in one paired sample (T and N), then a gene downregulation score of 1 was assigned.
[2]If the probe of the Infinium array was designed in the 5′-region of the gene, if $\Delta\beta$ ($\beta_T - \beta_N$) was −0.2 or less (DNA hypomethylation) and if ΔE ($E_T - E_N$) based on the expression microarray was 4 or more (overexpression) in one paired sample (T and N), then a gene upregulation score of 1 was assigned.

Among genes showing frequent genetic aberrations (genetic aberration score of 4 or more in Table 1), *GCN1L1* has recently been reported to be associated with the *CDK8* mediator complex, which includes *CDK8*, cyclin C (also known as *CCNC*), *MED12* and *MED13*.[25] *CDK8* directly regulates β-catenin-driven transcription[25] and human *CDK8* is known to be an oncogene that is amplified in a subset of colon cancers.[26] In addition, our quantitative RT-PCR analysis revealed a tendency for down regulation of β-catenin after knockdown of CDK8 by siRNA in RCC cell lines A-498 and ACHN (Supporting Information Fig. S4). These results are consistent with those of previous studies showing that knockdown of CDK8 in the human colon cancer cell line HCT116[27] and the human gastric cancer cell line SNU-638[28] resulted in significant reduction of β-catenin, indicating correlations between *CDK8* and the Wnt/β-catenin pathway.

The fly *MED12* and *MED 13* homologs, kohtalo and skuld, respectively activate Wnt/β-catenin target genes through direct interaction with the Wnt pathway component Pygopus.[29] However, *let-19* and *doy-22*, homologs of human *MED12* and *MED13*, respectively, in *Caenorhabditis elegans*, suppress the transcription of Wnt/β-catenin target genes.[30] Frequent mutation of human *MED12* has been reported in human uterine leiomyomas.[31] Deletion of the *CCNC* gene is frequently detected in human lymphoid malignancies[32] and sarcomas.[33] Wnt/β-catenin signaling is constitutively active in RCCs and activates their cell growth and metastasis.[34] However, unlike other human carcinomas, the incidence of mutation of exon 3 of the β-catenin gene is not so high in RCCs.[34] Analogously with other members of the *CDK8* mediator complex, mutations of *GCN1L1* may participate in renal carcinogenesis *via* Wnt/β-catenin signaling.

All 5 amino acid substitutions of the *GCN1L1* occurred within or near to Huntingtin protein, eEF3, protein phosphatase 2A and TOR (HEAT) repeats, which are crucial for protein-protein interaction[35] (Supporting Information Fig. S5). In addition, SIFT and PolyPhen-2 software predicted that amino acid substitutions due to mutations of the *GCN1L1* gene result in dysfunction of GCN1L1 protein (Table 1). The present study demonstrated not only a genetic aberration score of 5 for *GCN1L1*, but also a genetic aberration score of 3 for *MED12* and *CCNC* (Table 1). SIFT and PolyPhen-2 analyses have predicted that amino acid substitutions due to mutations of the *MED12* and *CCNC* genes also result in dysfunction of the proteins (Table 1). Taken together, the present data indicate that the function of the *CDK8* mediator complex may have been disturbed in 16% of the examined 67 RCCs. Genetic aberrations in members of the *CDK8* mediator complex may thus participate in the Wnt/β-catenin-related carcinogenetic pathway in clear cell RCCs.

MACF 1, a member of the plakin family of cytoskeletal linker proteins, regulates dynamic interactions between actin and microtubules to sustain directional cell movement.[36] *MACF1* is known to function in the Wnt signaling pathway through association with a complex containing axin, β-catenin, *GSK3β* and *APC* during mouse embryogenesis.[36] Somatic mutation of *MACF1* (Table 1) may also participate in the Wnt/β-catenin-related carcinogenetic pathway in clear cell RCCs. With respect

Table 3. Statistically significant GeneGo pathway maps revealed by MetaCore pathway analysis

		Involved genes		
Pathway	*P*-value	Genes	Entrez Gene ID	Multilayer-omics scoring (exome, methylome and transcriptome)
Cell adhesion_tight junctions	9.98×10^{-4}	*CLDN8*	9073	Downregulation score 20
		CLDN16	10686	Downregulation score 10
		CLDN19	149461	Downregulation score 19
Blood coagulation	1.26×10^{-3}	*VWF*	7450	Upregulation score 6
		F11	2160	Downregulation score 11
		FGG	2266	Upregulation score 6
Translation_non-genomic (rapid) action of androgen receptor	1.36×10^{-3}	*MTOR*	2475	Genetic score 4
		PTEN	5728	Genetic score 3
		EGF	1950	Downregulation score 10
Signal transduction_PTEN pathway	2.04×10^{-3}	*MTOR*	2475	Genetic score 4
		PTEN	5728	Genetic score 3
		EGF	1950	Downregulation score 10
Development_EGFR signaling *via* PIP3	7.04×10^{-3}	*PTEN*	5728	Genetic score 3
		EGF	1950	Downregulation score 10
Protein folding and maturation_-Bradykinin/ Kallidin maturation	1.34×10^{-2}	*KLK6*	5653	Downregulation score 5
		XPNPEP2	7512	Downregulation score 8
Transcription_receptor-mediated HIF regulation	1.95×10^{-2}	*MTOR*	2475	Genetic score 4
		PTEN	5728	Genetic score 3
Serotonin modulation of dopamine release in nicotine addiction	2.24×10^{-2}	*PTEN*	5728	Genetic score 3
		CHRM1	1128	Downregulation score 8
Signal transduction_AKT signaling	2.34×10^{-2}	*MTOR*	2475	Genetic score 4
		PTEN	5728	Genetic score 3
cAMP/ Ca(2+)-dependent Insulin secretion	2.34×10^{-2}	*PLCE1*	51196	Genetic score 3
		RYR2	6262	Genetic score 3
Immune response_interleukin-4 signaling pathway	2.45×10^{-2}	*MTOR*	2475	Genetic score 4
		GATA3	2625	Downregulation score 6
Role of alpha-6/beta-4 integrins in carcinoma progression	2.55×10^{-2}	*MTOR*	2475	Genetic score 4
		EGF	1950	Downregulation score 10
G-protein signaling_regulation of cAMP levels by muscarinic acetylcholine receptor	2.55×10^{-2}	*PLCE1*	51196	Genetic score 3
		CHRM1	1128	Downregulation score 8
Development_PIP3 signaling in cardiac myocytes	2.77×10^{-2}	*MTOR*	2475	Genetic score 4
		PTEN	5728	Genetic score 3

Table 3. Statistically significant GeneGo pathway maps revealed by MetaCore pathway analysis (Continued)

Pathway	*P*-value	Involved genes		
		Genes	Entrez Gene ID	Multilayer-omics scoring (exome, methylome and transcriptome)
Some pathways of EMT in cancer cells	3.22×10^{-2}	*MTOR*	2475	Genetic score 4
		EGF	1950	Downregulation score 10
Development_beta-adrenergic receptors signaling *via* cAMP	3.34×10^{-2}	*RYR2*	6262	Genetic score 3
		TNNC1	7134	Downregulation score 5
Development_IGF-1 receptor signaling	3.34×10^{-2}	*MTOR*	2475	Genetic score 4
		PTEN	5728	Genetic score 3
Translation _regulation of EIF4F activity	3.45×10^{-2}	*MTOR*	2475	Genetic score 4
		EGF	1950	Downregulation score 10
G-protein signaling_RAP2B regulation pathway	3.81×10^{-2}	*PLCE1*	51196	Genetic score 3
DNA damage_DNA-damage-induced responses	4.87×10^{-2}	*ATM*	472	Genetic score 3

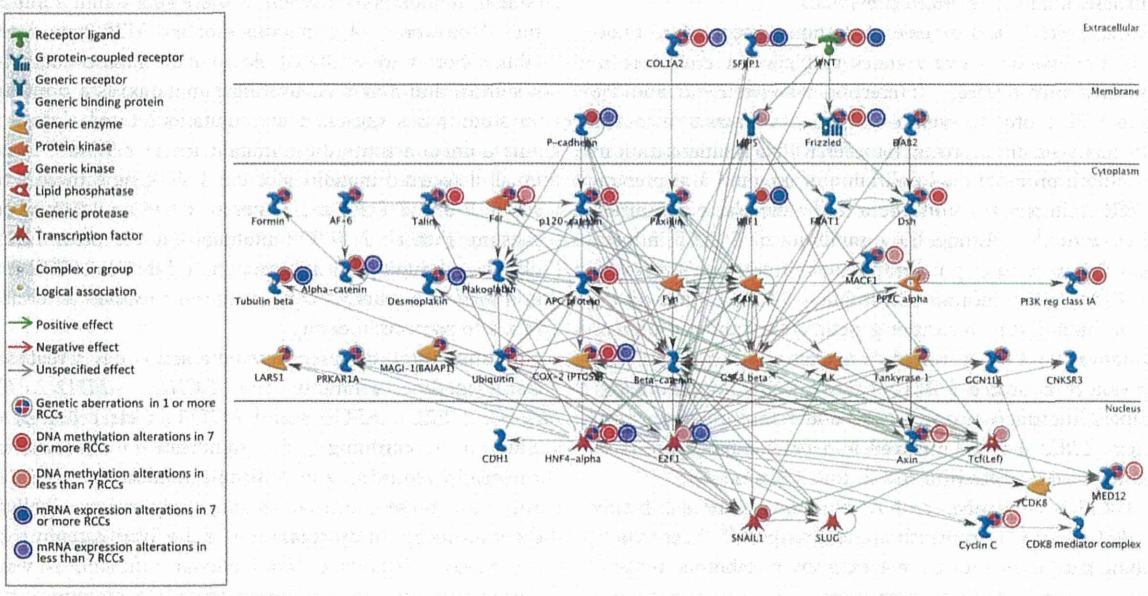

Figure 1. Genes for which a correlation with Wnt/β-catenin signaling was indicated by MetaCore pathway analysis. The numbers of genetic aberrations, DNA hyper- or hypo-methylation and/or increased or reduced mRNA expression (shown in Supporting Information Table S6) detected among the 67 examined RCCs are indicated schematically: legends are shown at the left of the panel. The 36 marked genes that showed genetic aberration, DNA methylation alterations and/or mRNA expression alterations in one or more RCCs were correlated with Wnt/β-catenin signaling.

to 29 RCCs for which transcriptome analysis was performed, mRNA expression levels of the targets genes of the Wnt/β-catenin signaling, such as *MYC*,[37] *MYCN*,[37] *IGF2*,[38] *POU5F1*,[39] *SOX9*,[40] *CYR61*,[41] *ENPP2*[42] and *MITF*,[43] tended to be higher in the 8 RCCs with mutations of any of the *GCN1L1, MED12, CCNC* and *MACF1* genes than in 21 RCCs without them (Supporting Information Table S10), indicating that such mutations may result in activation of Wnt/β-catenin signaling.

The downregulation score for the *SFRP1* gene was 6: reduced expression associated with DNA hypermethylation of *SFRP1* was frequent in clear cell RCCs. Members of the secreted frizzled-related protein (SFRP) family contain an N-terminal domain homologous to the cysteine-rich domain of the Wnt receptor Frizzled and lack a transmembrane region and the cytoplasmic domain required for transduction of signals into the cells.[44] This enables SFRPs to downregulate Wnt/β-catenin signaling by competing with Frizzled for Wnt binding *via* their cysteine-rich domain. Silencing of *SFRP1* due to DNA hypermethylation is known to result in activation of Wnt/β-catenin signaling.[44]

Since this study indicated possible alternative activation mechanisms (mutations of the *GCN1L1, MED12, CCNC* and *MACF1* genes and reduced expression of *SFRP1* due to DNA hypermethylation), we extensively examined Wnt/β-catenin signaling. MetaCore pathway analysis revealed that the 36 genes (marked in Fig. 1 and included in Supporting Information Table S6), which showed genetic aberration, DNA hypermethylation or hypomethylation and/or increased or reduced mRNA expression in one or more RCCs, are included in the Wnt/β-catenin signaling pathway. The present multilayer-omics analysis revealed that the Wnt/β-catenin signaling pathway may be of greater significance in renal carcinogenesis than was realized previously.

ERC2, which had a genetic aberration score of 4, is localized in presynaptic active zones and plays a critical role in neurotransmitter release.[45] Interaction between *ERC2* and the tandem PDZ protein syntenin-1, which is known to associate with many synaptic proteins, together with multimerization of *ERC2* both promote the localization of syntenin-1 at presynaptic *ERC2* clusters and contribute to the molecular organization of active zones.[45] Although the significance of *ERC2* in human cancers has remained unclear, frequent intragenic breaks in the *ERC2* gene indicated disruption of *ERC2* function in RCCs. In addition to recurrent genetic aberration, the present quantitative RT-PCR revealed frequent reduction of *ERC2* expression in clear cell RCCs relative to the corresponding N samples. Although frequent genetic and transcriptional inactivation of *ERC2* may be involved in renal carcinogenesis, further functional analysis of *ERC2* in RCCs is needed.

ABCA13 is a member of ATP-binding cassette sub-family A (*ABC1*) and a transmembrane transporter.[46] Xenobiotics, including anticancer drugs, are extensively metabolized by activation enzymes such as cytochromes *P450* and conjugation enzymes such as glutathione S-transferases or glucuronide transferases. Biotransformation represented by ABC transporters represents another important component of xenobiotic metabolism. In addition, ABC transporters play a crucial role in the development of resistance through efflux of anticancer agents from cancer cells.[46] The disease-free interval of patients with colorectal cancers treated by adjuvant chemotherapy is significantly shorter in patients with low *ABCA13* transcript levels.[47] In addition to recurrent genetic aberration (Table 1), the present quantitative RT-PCR revealed frequently reduced expression of *ABCA13* in RCCs relative to the corresponding N samples. Our findings suggest that it may be necessary to pay more attention to aberrations of *ABCA13* at both the genetic and expressional levels when deciding the indications for chemotherapy in patients with clear cell RCCs.

In Table 3 based on MetaCore pathway analysis, it is feasible that expression of *CLDN*s required for generating cation-selective paracellular channels[48] was reduced in clear cell RCCs, which lack the original absorptive function of the renal tubule. Moreover, *MTOR* mutations were highlighted as one of the major disrupters of multiple cell signaling during renal carcinogenesis: the *MTOR* gene participated in 10 (50%) of the 20 significant pathways in Table 3. The mammalian target of rapamycin (*mTOR*) encoded by the *MTOR* gene is a serine/threonine kinase that regulates cell growth, proliferation and autophagy.[49] *mTOR* inhibitors, such as rapamycin and its derivatives, are being introduced for targeted therapy of clear cell RCCs. Overactivation of *mTOR* is generally considered to be due to homozygous deletion of the *PTEN* tumor suppressor gene.[50] However, all 4 mutations of the *MTOR* gene detected in this cohort were located close to the kinase domain (data not shown) and may be activating mutations, as a previous *in vitro* study has suggested that mutations located close to the kinase domain activate the mutant form of *mTOR*.[50] In addition, all detected mutations of the *MTOR* gene showed a SIFT score of 0 and PolyPhen-2 scores of 0.998 or 0.999, strongly suggesting that all *MTOR* mutations affect protein function (Table 1 and Supporting Information Table S3). *MTOR* mutation may be a marker for predicting the sensitivity of clear cell RCCs to rapamycin therapy.

In summary, the present exome analysis has revealed frequent genetic aberrations of *GCN1L1, MED12, CCNC, MACF1, ERC2, ABCA13* and *MTOR* in clear cell RCCs. In addition to confirming the significance of aberrations of chromatin remodeling and histone modification-related proteins, the present multilayer-omics analysis has highlighted the significance of dysregulation of the Wnt/β-catenin signaling pathway including *CDK8* mediator function, as well as the need to pay closer attention to *MTOR* mutations, causing major disruption of cell signaling during renal carcinogenesis, in relation to chemosensitivity. Multilayer-omics analysis can be considered a powerful tool for revealing significant carcinogenetic pathways in human cancers.

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Druggable Oncogene Fusions in Invasive Mucinous Lung Adenocarcinoma

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