Comprehensive Exploration of Novel Chimeric Transcripts in Clear Cell Renal Cell Carcinomas Using Whole Transcriptome Analysis

Masahiro Gotoh,1† Hitoshi Ichikawa,2† Eri Arai,1 Suenori Chiku,3 Hiromi Sakamoto,2 Hiroyuki Fujimoto,4 Masaki Hiramoto,5 Takao Nammo,5 Kazuki Yasuda,5 Teruhiko Yoshida,2 and Yae Kanai1*  
1Division of Molecular Pathology, National Cancer Center Research Institute, Tokyo, Japan  
2Division of Genetics, National Cancer Center Research Institute, Tokyo, Japan  
3Science Solutions Division, Mizuho Information and Research Institute, Inc., Tokyo, Japan  
4Department of Urology, National Cancer Center Hospital, Tokyo, Japan  
5Department of Metabolic Disorder, Diabetes Research Center, National Center for Global Health and Medicine, Tokyo, Japan

The aim of this study was to clarify the participation of expression of chimeric transcripts in renal carcinogenesis. Whole transcriptome analysis (RNA sequencing) and exploration of candidate chimeric transcripts using the deFuse program were performed on 68 specimens of cancerous tissue (T) and 11 specimens of non-cancerous renal cortex tissue (N) obtained from 68 patients with clear cell renal cell carcinomas (RCCs) in an initial cohort. As positive controls, two RCCs associated with Xp11.2 translocation were analyzed. After verification by reverse transcription (RT)-PCR and Sanger sequencing, 26 novel chimeric transcripts were identified in 17 (25%) of the 68 clear cell RCCs. Genomic breakpoints were determined in five of the chimeric transcripts. Quantitative RT-PCR analysis revealed that the mRNA expression levels for the MMACHC, PTER, EPC2, ATXN7, FHIT, KIFAP3, CPEB1, MINPP1, TEX264, FAM107A, UPF3A, CDC16, MCC1, CPSF3, and ASAP2 genes, being partner genes involved in the chimeric transcripts in the initial cohort, were significantly reduced in 26 T samples relative to the corresponding 26 N samples in the second cohort. Moreover, the mRNA expression levels for the above partner genes in T samples were significantly correlated with tumor aggressiveness and poorer patient outcome, indicating that reduced expression of these genes may participate in malignant progression of RCCs. As is the case when their levels of expression are reduced, these partner genes also may not fully function when involved in chimeric transcripts. These data suggest that generation of chimeric transcripts may participate in renal carcinogenesis by inducing dysfunction of tumor-related genes.

INTRODUCTION

Clear cell renal cell carcinoma (RCC) is the most common histological subtype of adult kidney cancer (Ljungberg et al., 2011). In general, RCCs at an early stage are curable by nephrectomy. However, some RCCs relapse and metastasize to distant organs. Even though molecular targeting agents have been developed for treatment of RCCs, their effectiveness for relapsed or metastasized RCCs after nephrectomy is very limited. To improve prognostication and the effectiveness of targeting therapy in patients with RCCs, the molecular background of renal carcinogenesis should be further elucidated.

We and other groups have revealed both genetic and epigenetic events during renal carcinogenesis (Arai and Kanai, 2010). Especially, recent developments in high-throughput sequence capture methods and next-generation sequencing technologies have made exome sequencing technically feasible. Such whole exome analyses have revealed that renal carcinogenesis involves inactivation of histone-modifying genes such as SETD2 (Dalgliesh et al., 2010), KDM5C (Dalgliesh et al., 2010), UTX (van Haaften et al., 2009), and PBRM1...
Moreover, it is well known that clear cell RCCs are characterized by inactivation of the \textit{VHL} tumor suppressor gene encoding a component of the protein complex that possesses ubiquitin ligase E3 activity (Baldewijns et al., 2010). Frequent mutation of a further component of the ubiquitin-mediated proteolysis pathway gene, \textit{BAP1} (Guo et al., 2012), and \textit{VHL}-associated transcription elongation factor, \textit{TCEB1} (Sato et al., 2013), has also been demonstrated on the basis of exome analyses. However, only a limited number of reports have described next-generation sequencing-based whole transcriptome analysis (RNA sequencing) of RCCs, and the molecular background of renal carcinogenesis has not been fully elucidated.

**TABLE 1.** The Clinicopathological Parameters of Clear Cell Renal Carcinomas Belonging to the Initial and Second Cohorts

| Clinicopathological parameters                      | Initial cohort (\(n = 68\)) | Second cohort (\(n = 26\)) | \(P\)  |
|-----------------------------------------------------|------------------------------|-----------------------------|--------|
| Age (mean ± SD)                                      | 62.25 ± 11.00                | 57.12 ± 10.80               | 0.078* |
| Sex                                                  |                              |                             |        |
| Male                                                 | 49                           | 17                          | 0.616* |
| Female                                               | 19                           | 9                           |        |
| Tumor diameter (cm, mean ± SD)                       | 5.55 ± 3.21                  | 5.86 ± 2.84                 | 0.407* |
| Macropscopic configuration                           |                              |                             |        |
| Type 1                                               | 25                           | 13                          | 0.562* |
| Type 2                                               | 17                           | 5                           |        |
| Type 3                                               | 26                           | 8                           |        |
| Predominant histological grades\(^d\,\,^e\)          |                              |                             |        |
| G1                                                   | 36                           | 12                          | 0.696* |
| G2                                                   | 21                           | 8                           |        |
| G3                                                   | 9                            | 4                           |        |
| G4                                                   | 2                            | 2                           |        |
| Highest histological grades\(^f\)                    |                              |                             |        |
| G1                                                   | 5                            | 1                           | 0.105* |
| G2                                                   | 32                           | 6                           |        |
| G3                                                   | 16                           | 11                          |        |
| G4                                                   | 15                           | 8                           |        |
| Vascular involvement\(^e\)                           |                              |                             |        |
| Negative                                             | 38                           | 13                          | 0.649* |
| Positive                                             | 30                           | 13                          |        |
| Predominant growth pattern\(^n\)                     |                              |                             |        |
| Expansive                                            | 61                           | 24                          | 1.000* |
| Infiltrative                                         | 7                            | 2                           |        |
| Most aggressive growth pattern\(^f\)                 |                              |                             |        |
| Expansive                                            | 43                           | 21                          | 0.139* |
| Infiltrative                                         | 25                           | 5                           |        |
| Tumor necrosis                                       |                              |                             |        |
| Negative                                             | 51                           | 16                          | 0.212* |
| Positive                                             | 17                           | 10                          |        |
| Renal pelvic invasion\(^e\)                          |                              |                             |        |
| Negative                                             | 61                           | 23                          | 1.000* |
| Positive                                             | 7                            | 3                           |        |
| Distant metastasis\(^e\)                             |                              |                             |        |
| Negative                                             | 58                           | 24                          | 0.500* |
| Positive                                             | 10                           | 2                           |        |
| Pathological TNM stage\(^h\)                         |                              |                             |        |
| Stage I                                              | 33                           | 13                          | 0.531* |
| Stage II                                             | 4                            | 3                           |        |
| Stage III                                            | 19                           | 8                           |        |
| Stage IV                                             | 12                           | 2                           |        |

\(^{a}\)Mann–Whitney \(U\) test.  
\(^{b}\)Fisher’s exact test. No significant differences of clinicopathological parameters were observed between two cohorts.  
\(^{c}\)Macroscopic configuration was evaluated on the basis of previously described criteria (Arai et al., 2006).  
\(^{d}\)All the tumors were graded on the basis of previously described criteria (Fuhrman et al., 1982).  
\(^{e}\)If the tumor showed heterogeneity, findings in the predominant area were described.  
\(^{f}\)If the tumor showed heterogeneity, the most aggressive features of the tumor were described.  
\(^{g}\)The presence or absence of vascular involvement was examined microscopically on slides stained with hematoxylin-eosin and elastica van Gieson.  
\(^{h}\)All the tumors were classified according to the pathological Tumor-Node-Metastasis classification (Sobin et al., 2009).
TABLE 2. The 33 Chimeric Transcripts\(^a\) from the 61 Genes Verified by Reverse Transcription-PCR and Sanger Sequencing

| Exon boundaries\(^b\) | 5' partner gene | 3' partner gene | Flanking sequences\(^c\) |
|-----------------------|----------------|----------------|--------------------------|
| Subtype               | Sample         | Symbol         | RNA accession no.       | (chromosome, position) | Symbol | RNA accession no. | Distance (bp) | Flanking sequences\(^c\) |
| (A) Initial cohort    |                |                |                          |                        |        |                |              | In-frame transcripts |
| Clear K1              | ANTXR1         | NM_032208      | 17 (Ch 2, 69,420,547)   | GKN1                   | NM_019617 | 2 (Ch 2, 69,206,427) | 2,159,200 | TCTGGATGTCCAGCACGCACGGAGACACGG | + |
|                       | B4GALT2        | NM_001005417   | 3 (Ch 1, 44,450,537)    | ZSWIM5                 | NR_024270 | 1 (Ch 1, 45,765,959) | 13,196,023 | ATCTGCTTTGTGGATCTGGTAGTTT |
|                       | C9orf31        | NM_001193329   | 12 (Ch 9, 977,678,98)   | PRUNE2                 | NM_015225 | 19 (Ch 9, 7,992,295) | 185,388,282 | - |
|                       | CTNNB1         | NM_001005417   | 3 (Ch 1, 44,450,537)    | ZSWIM5                 | NR_024270 | 1 (Ch 1, 45,765,959) | 13,196,023 | ATCTGCTTTGTGGATCTGGTAGTTT |
|                       | ERBB2          | NM_001005417   | 15 (Ch 17, 378,721,96)  | GSK3B                  | NM_015225 | 19 (Ch 9, 7,992,295) | 185,388,282 | - |
|                       | MMACHC         | NM_001193329   | 12 (Ch 9, 977,678,98)   | PRUNE2                 | NM_015225 | 19 (Ch 9, 7,992,295) | 185,388,282 | - |
|                       | Clear K2       | DNAH11         | 79 (Ch 7, 21,934,167)   | RAPGEF5                | NM_012294 | 7 (Ch 7, 23,060,361) | 3,720,142 | - |
|                       | Clear K3       | PLOD2          | 1 (Ch 3, 145,756,47)    | CCNYL1                 | NM_001142300 | 4 (Ch 2, 20,860,213) | 4,238,760 | - |
|                       | Clear K4       | EP2C           | 2 (Ch 11, 625,497,26)   | CYP1A2                 | NM_000761 | 7 (Ch 15, 750,470) | 4,977,979 | - |
|                       | Clear K5       | ATXN7          | 5 (Ch 3, 43,981,57)     | HTMT1                  | NM_001166243 | 2 (Ch 3, 41,863,39) | 2,751,820 | - |
|                       | Clear K6       | POLR2G         | 2 (Ch 11, 625,497,26)   | CYP1A2                 | NM_000761 | 7 (Ch 15, 750,470) | 4,977,979 | - |
|                       | Clear K7       | AC010724       | 15 (Ch 8, 130,076,31)   | CPEB1                  | NM_001079533 | 8 (Ch 15, 821,804) | 1,077,177 | - |
|                       | Clear K8       | RIGBA          | 1 (Ch 11, 2,113,49)     | RP1–3-4P1.3.7          | NM_001079533 | 8 (Ch 15, 821,804) | 1,077,177 | - |
|                       | Clear K9       | SEMA6A         | 18 (Ch 5, 11,380,237)   | CAMK4                  | NM_000774 | 11 (Ch 5, 11,082,238) | 49,799,71 | - |
|                       | Clear K10      | RP1–3-2M19.1   | 10 (Ch 8, 8,900,587)    | MINPP1                 | NM_001178118 | 2 (Ch 10, 8,926,080) | 2,622,211 | - |
|                       | Clear K11      | TEX26          | 3 (Ch 3, 51,078,587)    | FAM107A                | NM_001076778 | 1 (Ch 3, 51,078,587) | 68,864,066 | - |
|                       | Clear K12      | UPF3A          | 5 (Ch 13, 11,502,104)   | CDC16                  | NM_001078645 | 15 (Ch 13, 11,502,104) | 2,474,621 | - |
|                       | Clear K13      | ASAP1          | 29 (Ch 8, 13,072,825)   | ADY8                   | NM_001115 | 10 (Ch 8, 13,186,204) | 7,892,24 | - |
### TABLE 2. (Continued)

| Subtype | Sample | Symbol | RNA accession no. | Exon (chromosome, position) | RNA accession no. | Exon (chromosome, position) | Distance (bp) | Flanking sequences | In-frame transcripts |
|---------|--------|--------|------------------|-----------------------------|------------------|-----------------------------|---------------|-------------------|----------------------|
| Clear   | K14    | DCUN1D1| NM_020640        | (ch 3, 182679014)           | MCCC1            | NM_020166                   | 18            | ch 3, 182735125   | 56111                |
| Clear   | K15    | CPSF3  | NM_016207        | (ch 2, 9576490)             | ASAP2            | NM_001135191                | 23            | ch 2, 9533611     | 42879                |
| Clear   | K16    | ADAMTS2| NM_014244        | (ch 5, 178770768)           | RPL11–78K2.34   | MCCC1                       | 159973        | ch 5, 178930741   | 159973               |
| Clear   | K17    | TPPP   | NM_007030        | (ch 5, 6778665)             | TERT             | NM_198253                   | 3             | ch 5, 1282739     | 604874               |
| (B) Positive controls |  |        |                  |                             |                 |                             |               |                   |                      |
| Xp11.2 K69 | EEF2  | NM_001961 | (ch 19, 3985376) |                             | ENHO             | NM_198573                   | 2             | ch 9, 34521854    |                      |
| NonO    | NM_00145408 | (ch X, 70517788) | TFE3              | NM_006521               | (ch X, 48891766) | 2162062          |                   |                    |
| RAG     | NM_003631 | (ch 10, 51993249) | BMSJ              | NM_014753               | (ch 10, 43287075) | 7806174          |                   |                    |
| RAGE    | NM_014226 | (ch 14, 102771299) | EMLJ              | NM_01008707             | (ch 14, 10031739) | 2454090          |                   |                    |
| Xp11.2 K96 | DPP6  | NM_001039350 | (ch 17, 153584819) |                             | ACTR38           | NM_020445       | 4             | ch 17, 152498706  | 108613               |
| TFE3    | NM_006521 | (ch X, 4889572) | RBM10             | NM_001204468           | (ch X, 47044545) | 1851268          |                   |                    |
| RBM10   | NM_001204468 | (ch X, 47041725) | TFE3              | NM_006521               | (ch X, 47045639) | 1853914          |                   |                    |

aChimeric transcripts (33) included the transcripts (MMACHC-BX004987.7 and TFE3-RBM10) consisting of the same partner gene sets with a different exon boundary or different transcriptional direction, and the transcripts sharing a partner gene, TFE3.

bNational Center for Biotechnology Information Database (Genome Build 37).

cThe exon boundaries are indicated by a vertical bar. Clear, clear cell renal cell carcinoma; Xp11.2, renal cell carcinoma associated with Xp11.2 translocation; ch, chromosome; +, positive; −, negative.
Human hematologic (Shima and Kitabayashi, 2011) and soft tissue malignancies (Cantile et al., 2013), prostatic adenocarcinoma (Tomlins et al., 2005), and distinct subtypes of lung adenocarcinoma (Soda et al., 2007; Kohno et al., 2012; Takeuchi et al., 2012) show “addiction” for gene fusion events. Although their incidence is low, fusion events involving the transcription factor TFE3 gene have been reported in RCCs: RCC associated with Xp11.2 translocation, which harbors TFE3 fusion, is considered to represent a distinct subtype according to the World Health Organization (WHO) classification (Eble et al., 2004). Moreover, fusion events including anaplastic lymphoma kinase (ALK), such as TMP3-ALK, EML4-ALK, and VCL-ALK fusion, have been reported in a distinct group of RCCs, including so-called “unclassified RCC” and papillary RCC in adults (Sugawara et al., 2012) and pediatric RCCs associated with the sickle cell trait (Debelenko et al., 2011; Mariño-Enríquez et al., 2011), based on fluorescence in situ hybridization (FISH) and immunohistochemistry. These findings have prompted us to perform comprehensive exploration of chimeric transcripts in renal carcinogenesis, whole transcriptome analysis was performed using tissue specimens of 68 clear cell RCCs in adults.

**MATERIALS AND METHODS**

**Patients and Tissue Samples**

The initial cohort subjected to whole transcriptome analysis comprised 68 samples of cancerous tissue (T) and 11 samples of non-cancerous renal cortex tissue (N) obtained from materials that had been surgically resected from 68 patients with primary clear cell RCCs. There were 49 men and 19 women with a mean (±standard deviation) age of 62.3 ± 11.0 years (range, 36 to 85 years). All patients underwent nephrectomy at the National Cancer Center Hospital, Tokyo, and had not received any preoperative treatment. Two expert pathologists specializing in genitourinary pathology, E.A. and Y.K., examined all histological slides and performed histological diagnosis in accordance with the WHO classification (Eble et al., 2004). All the tumors were graded on the basis of previously described criteria (Fuhrman et al., 1982) and classified according to the macroscopic configuration (Arai et al., 2006) and the pathological Tumor-Node-Metastasis (TNM) classification (Sobin et al., 2009). As a positive control for chimeric transcript detection, two T samples showing histological findings compatible with Xp11.2 translocation RCC based on the WHO criteria were also subjected to whole transcriptome analysis. For comparison, three T samples of papillary RCCs diagnosed in accordance with the WHO criteria were also subjected to whole transcriptome analysis.

The second cohort subjected to quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis comprised 26 paired T and N samples obtained from materials that had been surgically resected from 26 other patients with primary clear cell RCCs. These patients comprised 17 men and nine women with a mean (±standard deviation) age of 61.5 ± 11.0 years (range, 36 to 82 years).

**TABLE 3. Genomic Breakpoints of Chimeric Transcripts**

| Sample | Genomic position | Symbol | Chr | Genomic position | Symbol | Chr |
|--------|------------------|--------|-----|------------------|--------|-----|
| K6     | POLR2G           | 11     | 62530558 | CYP1A2         | 15     | 75045983 |
| K7     | AC010724.1       | 15     | 83207075 | CPEB1          | 15     | 83219352 |
| K9     | SEMA6A           | 5      | 11579806 | CAMK4          | 5      | 11082375 |
| K13    | ASAP1            | 8      | 131070249| ADcy8          | 8      | 131862252|
| K15    | CPSF3            | 2      | 9578689 | ASAP2          | 2      | 9532071 |

*aNational Center for Biotechnology Information Database (Genome Build 37).

bThe genomic breakpoints are indicated by a vertical bar.
Figure 1. Levels of mRNA expression for the partner genes involved in chimeric transcripts in 26 paired samples of tumorous tissue (T) and non-cancerous renal cortex tissue (N) in the second cohort. mRNA expression was analyzed using custom TaqMan Gene Expression Assays on the 7500 Fast Real-Time PCR System (Life Technologies) employing the relative standard curve method. The probes and PCR primer sets used are summarized in Supporting Information Table S6. Experiments were performed in triplicate for each sample-primer set, and the mean value for the three experiments was used as the CT value. All CT values were normalized to that of GAPDH in the same sample. Levels of mRNA expression for the MMACHC, PTER, EPC2, ATXN7, FHIT, KIFAP3, CPEB1, MINPP1, TEX264, FAM107A, UPF3A, CDC16, MCCC1, CPSF3, and ASAP2 genes were significantly reduced in T samples (shaded column) relative to N samples (white column). Bar, standard deviation.
### Table 4: Correlations Between Levels of mRNA Expression for Each of the Partner Genes Involved in Chimeric Transcripts in Tumorous Tissue Samples and Clinicopathological Parameters Reflecting Tumor Aggressiveness in the Second Cohort

| Clinicopathological Parameters | Number of Tumors | Expressiona | P     |
|--------------------------------|------------------|-------------|-------|
|                               |                  | MMACHC      | KIFAP3 | CPEB1 | TEX264 |
| **Macroscopic configuration**  |                  |             |       |       |        |
| Tumor necrosis                |                  |             |       |       |        |
| Positive                       | 5                | 0.160 ± 0.069 | 0.126 | 6.62 × 10⁻⁶ | 0.118 | 1.18 × 10⁻⁶ | 0.004 | 4.23 × 10⁻⁶ | 0.148 | 9.75 × 10⁻⁶ |
| Negative                       | 5                | 0.270 ± 0.0155 | 0.112 | 0.004 | 0.004 | 0.004 | 0.9978 | 0.0165 | 0.0165 | 0.9978 |
| Growth pattern                 |                  |             |       |       |        |
| Expansive                      |                  |             |       |       |        |
| Positive                       | 10               | 0.112 ± 0.040 | 0.180 | 6.62 × 10⁻⁶ | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Negative                       | 10               | 0.104 ± 0.010 | 0.104 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Distant metastasis             |                  |             |       |       |        |
| Negative                       |                  |             |       |       |        |
| Positive                       | 5                | 0.112 ± 0.0016 | 0.112 | 0.112 | 3.40 × 10⁻⁶ | 0.112 | 0.112 | 0.112 | 0.112 | 0.112 |
| Pathological TNM stage         |                  |             |       |       |        |
| Stage I                        |                  |             |       |       |        |
| Positive                       | 9                | 0.170 ± 0.015 | 0.170 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Negative                       | 9                | 0.170 ± 0.015 | 0.170 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Stage II                       |                  |             |       |       |        |
| Positive                       | 9                | 0.104 ± 0.010 | 0.104 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Negative                       | 9                | 0.104 ± 0.010 | 0.104 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Stage III                      |                  |             |       |       |        |
| Positive                       | 9                | 0.104 ± 0.010 | 0.104 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Negative                       | 9                | 0.104 ± 0.010 | 0.104 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Stage IV                       |                  |             |       |       |        |
| Positive                       | 9                | 0.104 ± 0.010 | 0.104 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |
| Negative                       | 9                | 0.104 ± 0.010 | 0.104 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 |

**Genes, Chromosomes & Cancer DOI 10.1002/gcc**
Renal pelvic invasion
Growth pattern
Vascular involvement

All the tumors were classified according to the pathological Tumor-Node-Metastasis classification (Sobin et al., 2009). Although no significant correlation between expression of any of the 26 chimeric transcripts and clinicopathological parameters was observed in the initial cohort (Supporting Information Fig. S1). These findings were considered to be the hallmark of clear cell RCCs in the initial and second cohorts. The clinicopathological parameters of RCCs belonging to the initial and second cohorts are summarized in Table 1.

Tissue specimens were taken and frozen immediately after surgical removal, and thereafter stored in liquid nitrogen until use. These tissue specimens were provided by the National Cancer Center Biobank, Tokyo. This study was approved by the Ethics Committees of the National Cancer Center and National Center for Global Health and Medicine, Tokyo, and was performed in accordance with the Declaration of Helsinki. All the patients provided written informed consent prior to inclusion in the study.

Whole Transcriptome Analysis

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA). A total of 84 (73 T and 11 N) samples in the initial cohort were subjected to whole transcriptome analysis. Sequencing libraries were prepared from 1.0 to 2.5 µg of total RNA using an mRNA-Seq Sample Prep Kit or a TruSeq RNA Sample Prep Kit (Illumina), according to the manufacturer’s standard protocols. An mRNA-Seq Sample Prep Kit was used for libraries of 35 (30 T and 5 N) samples, and these libraries were prepared using a procedure including a gel purification step, in which a

*Average mRNA levels/GAPDH ± standard deviation.

*Macroscopic configuration was evaluated on the basis of previously described criteria (Arai et al., 2006).

*Kruskal–Wallis test. P values of < 0.05 are underlined.

*All the tumors were classified according to the pathological Tumor-Node-Metastasis classification (Sobin et al., 2009). Although no significant correlation between expression of any of the 26 chimeric transcripts and clinicopathological parameters was observed in the initial cohort (Supporting Information Table S4), downregulation of mRNA levels for each of the partner genes did show significant correlations with the above clinicopathological parameters in the second cohort.

| Clinicopathological parameters | Number of Tumors | FAM107A | CDC16 | CPSF3 | ASAP2 |
|-------------------------------|------------------|---------|-------|-------|-------|
| Expression | P | Expression | P | Expression | P | Expression | P |
| Type 2 | 5 | 0.0986 ± 0.0779 | 0.0584 ± 0.0377 | 0.0260 ± 0.0151 | 0.0218 ± 0.0191 |
| Type 3 | 8 | 0.203 ± 0.242 | 0.0926 ± 0.0753 | 0.0465 ± 0.0286 | 0.0250 ± 0.0227 |
| Histological grades | | | | | |
| G1 | 1 | 0.685 | 2.14 × 10⁻²⁵ | 0.172 | 1.28 × 10⁻²⁵ | 0.0613 | 1.30 × 10⁻²⁵ | 0.112 | 1.93 × 10⁻²⁵ |
| G2 | 6 | 0.209 ± 0.140 | 0.113 ± 0.083 | 0.0575 ± 0.0335 | 0.0399 ± 0.0200 |
| G3 | 11 | 0.313 ± 0.197 | 0.016 ± 0.049 | 0.0464 ± 0.0236 | 0.0411 ± 0.0252 |
| G4 | 8 | 0.100 ± 0.129 | 0.0475 ± 0.0206 | 0.0256 ± 0.0118 | 0.0122 ± 0.0068 |
| Vascular involvement | | | | | |
| Negative | 13 | 0.258 ± 0.182 | 3.11 × 10⁻¹⁸ | 0.123 ± 0.063 | 2.56 × 10⁻¹⁸ | 0.0518 ± 0.0271 | 1.13 × 10⁻¹⁸ | 0.0508 ± 0.030 | 7.95 × 10⁻⁹ |
| Positive | 13 | 0.217 ± 0.226 | 0.0696 ± 0.0458 | 0.0344 ± 0.0214 | 0.0185 ± 0.0203 |
| Growth pattern | | | | | |
| Expansive | 21 | 0.255 ± 0.211 | 4.47 × 10⁻¹⁸ | 0.0975 ± 0.0608 | 8.01 × 10⁻¹⁸ | 0.0437 ± 0.0263 | 9.00 × 10⁻¹⁸ | 0.0372 ± 0.0316 | 4.09 × 10⁻¹⁸ |
| Infiltrative | 5 | 0.166 ± 0.159 | 0.0914 ± 0.0662 | 0.0409 ± 0.0246 | 0.0240 ± 0.0211 |
| Tumor necrosis | | | | | |
| Negative | 16 | 0.317 ± 0.205 | 2.24 × 10⁻¹⁸ | 0.121 ± 0.063 | 5.02 × 10⁻¹⁸ | 0.0353 ± 0.0266 | 1.44 × 10⁻¹⁸ | 0.0475 ± 0.0317 | 2.77 × 10⁻⁹ |
| Positive | 10 | 0.110 ± 0.117 | 0.0567 ± 0.0278 | 0.0266 ± 0.0115 | 0.0142 ± 0.0086 |
| Renal pelvic invasion | | | | | |
| Negative | 23 | 0.259 ± 0.205 | 7.85 × 10⁻¹⁸ | 0.100 ± 0.063 | 3.52 × 10⁻¹⁸ | 0.0453 ± 0.0263 | 3.95 × 10⁻¹⁸ | 0.0368 ± 0.0313 | 4.42 × 10⁻¹⁸ |
| Positive | 3 | 0.072 ± 0.0462 | 0.0650 ± 0.0128 | 0.0263 ± 0.0073 | 0.0179 ± 0.0058 |
| Distant metastasis | | | | | |
| Negative | 24 | 0.256 ± 0.199 | 5.54 × 10⁻¹⁸ | 0.102 ± 0.059 | 2.46 × 10⁻¹⁸ | 0.0457 ± 0.0249 | 1.23 × 10⁻¹⁸ | 0.0363 ± 0.0307 | 3.94 × 10⁻¹⁸ |
| Positive | 2 | 0.0165 ± 0.0030 | 0.0245 ± 0.0007 | 0.0125 ± 0.0019 | 0.0151 ± 0.0005 |
| Pathological TNM stage | | | | | |
| Stage I | 13 | 0.246 ± 0.152 | 2.72 × 10⁻¹⁸ | 0.109 ± 0.053 | 4.59 × 10⁻¹⁸ | 0.0445 ± 0.0279 | 9.67 × 10⁻¹⁸ | 0.0404 ± 0.0279 | 1.86 × 10⁻¹⁸ |
| Stage II | 3 | 0.209 ± 0.167 | 0.0513 ± 0.0307 | 0.0324 ± 0.0170 | 0.0103 ± 0.0082 |
| Stage III | 8 | 0.290 ± 0.284 | 0.110 ± 0.071 | 0.0326 ± 0.0200 | 0.0394 ± 0.0376 |
| Stage IV | 2 | 0.0165 ± 0.0030 | 0.0245 ± 0.0007 | 0.0125 ± 0.0019 | 0.0151 ± 0.0005 |
fraction of 250–300 bp (insert size: 150–200 bp) was collected. A TruSeq RNA Sample Prep Kit was used for libraries of the other 49 (43 T and 6 N) samples, and these libraries were prepared without gel purification. The resulting libraries were subjected to paired-end sequencing of 50-base reads on a GAIIx or HiSeq2000 sequencer (Illumina).

Detection of Chimeric Transcripts

To avoid multiple counting of each chimeric transcript, RNA sequencing data were used after removal of paired-end reads with the identical nucleotide sequence, which had probably been derived from PCR duplicates during library preparation. For prediction of chimeric transcripts, the deFuse program version 0.4.3 was used (McPherson et al., 2011). After applying default filtering of this program, potential alternative splicing and read-through products that the program predicted were eliminated, and candidates that had exon boundary junctions were selected. Finally, we discarded candidates that were also predicted from the data of 11 N samples.

RT-PCR and Sanger Sequencing

cDNA was reverse-transcribed from the total RNA (500 ng) of the initial cohort samples, in which candidate chimeric transcripts were detected by whole transcriptome analysis, using random primers and Superscript III RNase H Reverse Transcriptase (Life Technologies). cDNA (corresponding to 10 ng total RNA) was subjected to PCR amplification using an optimal DNA polymerase among AmpliTaq Gold DNA Polymerase (Life Technologies), HotStar Taq DNA polymerase (Qiagen, Hilden, Germany) or KAPA Taq DNA Polymerase (KAPA Biosystems, Woburn, MA). The PCR products were separated electrophoretically on 2% agarose gel and stained with ethidium bromide to confirm that specific products of the size estimated on the basis of whole transcriptome analysis were obtained, and that no nonspecific products appeared on amplification. The PCR products were then directly sequenced in both directions using the same primers with the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3130xl DNA Sequencer (Life Technologies).

Genomic PCR and Sanger Sequencing

High-molecular-weight genomic DNA was extracted from the initial cohort samples, in which candidate chimeric transcripts were verified by the above RT-PCR and Sanger sequencing, using phenol–chloroform followed by dialysis. Genomic...
DNA (10 ng) was subjected to PCR amplification using an optimal DNA polymerase among AmpliTaq Gold DNA Polymerase, Platinum Taq DNA-polymerase high fidelity (Life Technologies), HotStar Taq DNA polymerase (Qiagen) or KAPA Taq DNA Polymerase (KAPA Biosystems). The PCR products were separated electrophoretically on 1% agarose gel and stained with ethidium bromide to confirm that no nonspecific products appeared on amplification. The PCR products were then directly sequenced in both directions using the same primers with the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3130xl DNA Sequencer (Life Technologies).

**Quantitative RT-PCR Analysis**

cDNA was reverse transcribed from total RNA (500 ng) of the 26 paired T and N samples of the second cohort using random primers and Superscript III RNase H Reverse Transcriptase (Life Technologies). mRNA expression was analyzed using custom TaqMan Gene Expression Assays and TaqMan Fast Advanced Master Mix (Life Technologies) on a 7500 Fast Real-Time PCR System (Life Technologies) employing the relative standard curve method. Experiments were performed in triplicate for each sample-primer set, and the mean value for the three experiments was used as the CT value. All CT values were normalized to that of GAPDH in the same sample.

**Statistics**

Differences in clinicopathological parameters between the initial and second cohorts were assayed by Mann–Whitney U test and Fisher’s exact test. Differences in the levels of mRNA expression between N and T samples were examined by Mann–Whitney U test. Correlations between levels of mRNA expression and clinicopathological parameters were assayed by Kruskal–Wallis test and Mann–Whitney U test. Receiver operating characteristic (ROC) curves were generated for the levels of mRNA expression of each partner gene involved in the chimeric transcripts, and the thresholds were set at the top left corner of the graph. Subsequently, the impact of chimeric transcript expression and downregulation of mRNA levels for each partner gene on patient outcome was analyzed by the Kaplan–Meier method using the set thresholds and the log-rank test. Differences at $P < 0.05$ were considered to be significant.

**RESULTS**

**Identification of Novel Chimeric Transcripts in RCCs of the Initial Cohort**

We performed RNA sequencing of 68 T samples (K1 to K68) and 11 N samples in the initial cohort, and a T sample (K69) showing histological findings compatible with Xp11.2 translocation RCC. At least 30,000,000 reads (average read count 50,000,000) were obtained for each sample. The deFuse program version 0.4.3 (McPherson et al., 2011) provided 3,746 fusion gene candidates from the data obtained using the 69 T samples by applying default filtering. From those candidates, 95 were extracted by eliminating potential alternative splicing and read-through products that the program predicted, and by selecting candidates that had exon boundary junctions. Next, candidates that were predicted even from the data obtained using the 11 N samples were discarded, and finally 35 candidates were obtained. Three candidates were abandoned because of difficulty with the primer design and shortage of samples, and then RT-PCR analysis was performed for the 32 candidates in the same T sample. The PCR and sequencing primers used are shown in Supporting Information Table S3.

After a T sample (K96) of Xp11.2 translocation RCC and three T samples of papillary RCCs (K97 to K99) had been additionally analyzed for comparison, expression of 33 fusion transcripts (including two transcripts [MMACHC-BX004987.7 and TFE3-RBM10] consisting of the same partner gene sets with a different exon boundary or a different transcriptional direction and three transcripts sharing a partner gene, TFE3) from the 61 genes was finally verified by RT-PCR, and the exon boundaries and flanking sequences were determined by Sanger sequencing analysis (Table 2 and Supporting Information Fig. S2).

Previously reported in-frame fusion transcripts including TFE3 (Table 2B; Clark et al., 1997), which are attributable to translocation of the X chromosome, were detected in samples K69 and K96 showing histological findings compatible with Xp11.2 translocation RCC, indicating the reliability of our study. Other than TFE3 fusion transcripts, three additional transcripts (EEF2-ENHO, PARG-BMS1, and RAGE-EML1, Table 2B) and one additional transcript (DPP6-ACTR3B, Table 2B), which have never been reported in RCCs, were also detected in the K69 and K96, respectively. NONO-TFE3, PARG-BMS1, RAGE-EML1, RBM10-TFE3, and DPP6-ACTR3B transcripts...
were predicted to generate in-frame chimeric proteins. These observations of additional chimeric transcripts in K69 and K96 were different from the previously reported characteristics of RCCs associated with Xp11.2 translocation [Philueger et al. (2013) reported that expression of the TMED6-COG8 chimeric transcript and higher expression levels of the EEF1A2 and CNTN3 genes characterize RCCs associated with Xp11.2 translocation]. K69 showed grade 3 histology, vascular involvement, and tumor necrosis in surgically resected materials, and the patient developed lymph node metastasis 6 months after surgery, whereas K96 showed grade 3 histology. Such phenotypes, especially those of K69, which are more aggressive than those generally described for RCCs with Xp11.2 translocation (Eble et al., 2004), may be attributable to expression of multiple additional chimeric transcripts. Conversely, in three papillary RCCs (K97 to K99) analyzed for comparison, no chimeric transcript was detected.

All 26 chimeric transcripts detected in the initial cohort of clear cell RCCs (Table 2A) have never been reported previously. Even though chimeric transcripts involving the FHIT and TERT genes have recently been sequenced by The Cancer Genome Atlas (TCGA, The Cancer Genome Atlas Research Network, 2013), the partner gene of FHIT (FAM172A), and that of TERT, PDCD6, listed in TCGA each differed from those (ATXN7 and TPPP) in the present study. Each of the detected chimeric transcripts was expressed in a single clear cell RCC. ANTXR1-GKN1, ERBB2-LTB4, POLR2G-CYP1A2, AC010724.1-CPEB1, and CPSF3-ASAP2 chimeric transcripts were predicted to generate in-frame chimeric proteins, whereas other chimeric transcripts resulted in a premature stop codon in the 3'-partner gene or were generated in the untranslated regions.

The chimeric transcripts were expressed in 17 clear cell RCCs in the initial cohort [17/68 (Table 2), 25%]. Samples K1 and K5 had multiple chimeric transcripts (Table 2). No significant correlation between expression of any of 26 chimeric transcripts and clinicopathological parameters was observed in the initial cohort (Supporting Information Table S4). However, when examined individually, each clear cell RCC with chimeric transcripts showed tumor aggressiveness: e.g., K11 carrying a TEX264-FAMI07A chimeric transcript showed a type 3 macroscopic configuration and K15 carrying a CPSF3-ASAP2 chimeric transcript showed a type 3 macroscopic configuration, grade 4 histology, vascular involvement, an invasive growth pattern, and tumor necrosis. Moreover, expression of any of the 26 chimeric transcripts was inversely correlated with the cancer-free survival rate of patients in the initial cohort (the period covered from 42 to 4,783 days [mean, 2,015 days]; log-rank test, \( P = 3.19 \times 10^{-5} \); Fig. 2).

Identification of Genomic Breakpoints in RCCs of the Initial Cohort

Long-range genomic PCR and Sanger sequencing were performed for 17 clear cell RCCs (K1 to K17) harboring chimeric transcripts using the primers shown in Supporting Information Table S5. Genomic breakpoints for five chimeric transcripts, POLR2G-CYP1A2, AC010724.1-CPEB1, SEMA6A-CAMK4, ASAP1-ADCY8, and CPSF3-ASAP2, were successfully revealed, but the genomic PCR failed for the other transcripts. The genomic breakpoints for these five chimeric transcripts are summarized in Table 3.

Levels of mRNA Expression for the Genes Involved in Chimeric Transcripts

The levels of mRNA expression for 20 representative partner genes involved in chimeric transcripts in the initial cohort were quantitatively examined in 26 paired T and N samples in the second cohort. The probes and PCR primer sets used are shown in Supporting Information Table S6.

The levels of mRNA expression for the MMACHC, PTER, EPC2, ATXN7, FHIT, KIFAP3, CPEB1, MINPP1, TEX264, FAM107A, UFP3A, CDC16, MCCC1, CPSF3, and ASAP2 genes were significantly reduced in T samples relative to the corresponding N samples (Fig. 1, Mann–Whitney U test, \( P = 3.38 \times 10^{-12} \), \( P = 9.04 \times 10^{-7} \), \( P = 8.08 \times 10^{-3} \), \( P = 6.21 \times 10^{-4} \), \( P = 2.71 \times 10^{-11} \), \( P = 9.46 \times 10^{-8} \), \( P = 2.18 \times 10^{-5} \), \( P = 1.03 \times 10^{-2} \), \( P = 2.47 \times 10^{-9} \), \( P = 9.90 \times 10^{-5} \), \( P = 2.53 \times 10^{-3} \), \( P = 1.89 \times 10^{-4} \), \( P = 1.62 \times 10^{-5} \), \( P = 1.77 \times 10^{-5} \), and \( P = 7.17 \times 10^{-9} \), respectively). The levels of mRNA expression for the MMACHC, PTER, EPC2, ATXN7, FHIT, KIFAP3, CPEB1, TEX264, FAM107A, CDC16, CPSF3, and ASAP2 genes in T samples in the second cohort were significantly correlated with clinicopathological parameters reflecting tumor aggressiveness, such as invasive macroscopic configuration, higher histological grades, vascular involvement, invasive growth pattern, tumor necrosis, renal pelvic invasion, distant metastasis,
and higher TNM stages (Table 4). Moreover, mRNA levels for the ATXN7 (P = 1.96 × 10^{-3}), KIFAP3 (P = 4.72 × 10^{-2}), FAM107A (P = 6.14 × 10^{-3}), and UPF3A (P = 4.19 × 10^{-2}) genes in T samples were inversely correlated with the cancer-free survival rate, whereas those for the EPC2 (P = 4.44 × 10^{-2}), FHIT (P = 3.22 × 10^{-2}), CPEB1 (P = 9.45 × 10^{-3}), and ASAP2 (P = 2.24 × 10^{-2}) genes were inversely correlated with the overall survival rate in the second cohort (the period covered ranged from 88 to 5,207 days [mean, 3,038 days], the log-rank test; Fig. 2).

**DISCUSSION**

To comprehensively explore chimeric transcripts in clear cell RCCs, whole transcriptome analysis was performed using tissue specimens. The significance of generation of chimeric transcripts has not been fully elucidated in adult solid tumors other than well-studied exceptions, such as sarcomas and adenocarcinomas of the prostate and the lung. Although previous reports of fusion events involving the ALK gene based on FISH and immunohistochemistry have been restricted to nonclear cell RCCs (Sugawara et al., 2012), when comprehensively explored using next-generation sequencing technology, chimeric transcripts were detected in 25% (17/68) of the clear cell RCCs. In some RCCs (K1 and K5), multiple chimeric transcripts were observed. Moreover, the genomic breakpoints revealed for five chimeric transcripts in clear cell RCCs indicate that such transcripts have actually arisen through genomic rearrangement. Gene fusion events may thus play a greater role in renal carcinogenesis than previously anticipated. Conversely, mechanisms other than genomic rearrangements (Yuan et al., 2013), e.g., trans-splicing (Li et al., 2008), may generate chimeric transcripts in which genomic breakpoints are not revealed.

The WHO classification defines RCC associated with Xp11.2 translocation, which involves TFE3 fusion, as a distinct subtype (Eble et al., 2004). Diagnosis of RCC associated with Xp11.2 translocation depends on detection of TFE3 protein overexpression using immunohistochemistry or detection of gene fusion using FISH and/or RT-PCR analysis (Green et al., 2013; Rao et al., 2013). The procedure for final diagnosis of RCC associated with Xp11.2 translocation differs from that for other RCC subtypes, such as clear cell RCC, papillary RCC, and chromophobe RCC, which generally can be diagnosed on the basis of histological observation. As RCC associated with Xp11.2 translocation and other RCCs were lumped into the same category as the RCC subtypes, the final diagnosis of RCC subtypes could not be made based solely on conventional histological examination of surgically resected or biopsy specimens. As the present comprehensive study demonstrated multiple chimeric transcripts in various RCCs, it seems that the use of Xp11.2 translocation as the only criterion for defining a distinct subtype of RCC may not be a rational approach. The classification of RCC subtypes should therefore be revised after a more comprehensive appraisal of correlations between histological features and genetic background.

All 26 chimeric transcripts detected in the initial cohort were novel chimeric transcripts that have never been reported previously in RCCs. However, only five of them were predicted to generate in-frame chimeric proteins in clear cell RCCs. Expression microarray analysis did not necessarily suggest prominent overexpression of in-frame chimeric transcripts in the initial cohort (data not shown). Moreover, in-frame chimeric transcripts observed in clear cell RCCs do not necessarily result in constitutive activation of protein kinases, which frequently cause addiction for gene fusion events. Conversely, many genes for which reduced expression and/or tumor suppressive function have been reported in human cancers were included in chimeric transcripts observed in the initial cohort. Therefore, we examined the levels of mRNA expression for 20 representative genes involved in chimeric transcripts in the second cohort (Supporting Information Table S6) and revealed significantly reduced mRNA expression of the MMACHC, PTER, EPC2, ATXN7, FHIT, KIFAP3, CPEB1, MINPP1, TEX264, FAM107A, UPF3A, CDC16, MCCC1, CPSF3, and ASAP2 genes in T samples in the second cohort (Fig. 1).

It has been reported that reduced expression of the MMACHC gene, which participates in intracellular trafficking of cobalamin, can result in increased tumorigenicity and methionine dependence of cancer cells (Loewy et al., 2009). Although its implication in human cancers has been unclear, the PTER gene was first cloned as a rat homolog of bacterial phosphotriesterase, and its expression in the normal proximal tubules of the kidney has been reported (Davies et al., 1997). Single nucleotide polymorphism (SNP) of the EPC2 gene has been reported to be associated with response to gemcitabine in human cancer cell lines (Jarjanazi et al., 2008). SNP of the ATXN7 gene, which
encodes a subunit of the GCN5 histone acetyltransferase-containing coactivator complex (Helminger et al., 2006), is reportedly associated with susceptibility to breast cancer (Milne et al., in press). The fragile FHit gene, encompassing the chromosomal fragile site FRA3B, is a target of DNA damage-induced cancer initiation and progression through modulation of genomic stability (Karras et al., 2014). KIFAP3 is colocized with KIF3, which participates in subcellular transport of several cancer-related proteins including beta-catenin and cadherins (Tanuma et al., 2009). Down regulation of CPEB1, which participates in the regulation of mRNA translation and processing of the 3' untranslated region (Bava et al., 2013), has been reported in human cancers (Caldeira et al., 2012). As has been reported for the PTEN gene, somatic mutation and germline variants of the MINPP1 gene, located in proximity to PTEN in 10q23.3, have been reported in patients with follicular thyroid tumors (Gimm et al., 2001). FAM107A was first identified in a commonly deleted region in 3p21 in RCCs (Wang et al., 2000), and transfection of this gene induces growth suppression and apoptosis of FAM107A-negative cancer cell lines (Wang et al., 2000; Liu et al., 2009). UPF3A is a crucial factor of nonsense-mediated decay, an RNA decay pathway that downregulates aberrant mRNAs (Chan et al., 2009). CDC16 is a component of the Anaphase Promoting Complex/Cyclosome (APC/C), which governs cell cycle progression and has crucial functions in maintaining genomic integrity and tumorogenesis (Zhang et al., 2014). Genetic imbalance at the MMCC1 gene locus has recently been reported in clinical specimens of oral squamous cell carcinoma (Ribeiro et al., 2014). CPSF3 is required for site-specific endonucleolytic cleavage and poly (A) addition (Keller and Minvielle-Sebastia, 1997) and directly interacts with (Zhu et al., 2009) tumor suppressor gene product CSR1 (Yu et al., 2006). The src homology 3 domain of the paxillin-binding protein (Kondo et al., 2000; Coutinho-Camillo et al., 2006), ASAP2, directly interacts with the SAMP repeat region of the APC tumor suppressor gene (Matsui et al., 2008).

Although the TEX264 gene has been simply identified as one of the protein-encoding open reading frames deposited in a database (Lamesch et al., 2007), the above characteristics of each of the partner genes suggest that down-regulation of the MMACHC, PTEN, EPC2, ATXN7, FHIT, KIFAP3, CPEB1, MINPP1, FAM107A, UPF3A, CDC16, MMCC1, CPSF3, and ASAP2 genes may participate in renal carcinogenesis. Moreover, the levels of mRNA expression for many of the partner genes in T samples were significantly correlated with the clinicopathological aggressiveness of RCCs (Table 4) and were inversely correlated with the cancer-free and/or overall survival rates of patients with clear cell RCCs (Fig. 2), indicating that such reduced expression may continue to play a role in multistage malignant progression during renal carcinogenesis.

Even if the same chimeric transcripts detected in the initial cohorts had been expressed in the second cohort, quantitative RT-PCR analysis for each partner gene would not have evaluated chimeric transcripts lacking target exons (Supporting Information Table S6). Therefore, to reveal the presence or absence of the same chimeric transcripts detected in the initial cohort, RT-PCR analysis using total RNA samples and the primer sets indicated in Supporting Information Table S3 and long-range PCR analysis using genomic DNA samples and the primer sets described in Supporting Information Table S5 were performed in the second cohort. These analyses did not detect the same chimeric transcripts in the second cohort (data not shown). As all detected chimeric transcripts were expressed only in a single clear cell RCC in the initial cohort, it is possible that the same chimeric transcripts may have been absent in the second cohort. Downregulation of mRNA levels for each of the genes described in Figure 1 in the second cohort would have been attributable to mechanisms other than expression of chimeric transcripts, such as gene deletion, DNA methylation status around the promoter regions and/or alterations in the expression levels, and accessibility of transcription factors. In fact, silencing of the MMACHC (Loewy et al., 2009) and CPEB1 (Caldeira et al., 2012) genes due to DNA methylation, and gene deletion and DNA methylation of FHIT (Karras et al., 2014), have been reported in human cancers. However, further studies are needed to reveal the mechanisms responsible for downregulation of each of the partner genes in the second cohort.

Conversely, it is feasible that dysfunction of each partner gene is induced by generation of chimeric transcripts in the initial cohort of clear cell RCCs, as such mechanisms of tumor suppressor gene functional impairment have been reported in adult malignancies such as acute myeloid leukemia (McNerney et al., 2013). Even though prominent overexpression and/or constitutive activation of growth factors and/or protein kinases due to
gene fusion events are rare, generation of chimeric transcripts may participate in tumor carcinogenesis through dysfunction of tumor-related genes.

REFERENCES

Arai E, Kanai Y. 2010. Genetic and epigenetic alterations during renal carcinogenesis. Int J Clin Exp Pathol 4:58–73.

Arai E, Kanai Y, Ushijima S, Fujimoto H, Mukai K, Hirohashi S. 2010. Genetic and epigenetic alterations during renal cell carcinoma. J Pathol 221:125–138.

Bava FA, Eliscovich C, Ferreira PG, Miura R, Arai E, Kanai Y. 2010. Genetic and epigenetic alterations during renal carcinoma reveals inactivation of histone-modifying enzymes. Int J Cancer 126:369–378.

Budillon MG, van Vlodrop JJ, Vermeulen PB, Soetekouw PM, van Engeland M, de Bruiine AP. 2010. VHIL and HIF signalling in renal cell carcinoma. J Pathol 221:125–138.

Baya FA, Eliscovich C, Ferreira PG, Mihana B, Ben-Dov C, Guigo R, Valcarcel J, Medneze R. 2013. CPEB1 coordinates alternative 3'-UTR formation with translational regulation. Nature 495:121–125.

Calderon J, Simeone-Conrea J, Paredes J, Pinto MT, Souza S, Corso G, Marrelli D, Roviole F, Pereira PS, Weil D, Oliveira C, Casares F, Sernau R. 2012. CPEB1, a novel gene silenced in gastric cancer: A Drosophila approach. Gut 61:1115–1123.

Canzoneri A, Marra I, Franco R, Accetta P, Pizuvino S, De Chiaro A, Bott G. 2013. Molecular detection and targeting of EWSR1 fusion transcripts in soft tissue tumors. Med Oncol 30:412.

Chan WK, Bhalla AD, Le Hir H, Nguyen LS, Huang L, Gao S, Tang A, Hu X, Huang Y, Jia W, Li Z, He Y, Poon C, Hsu JY, Liu Z, Wu Y, Zhao H, Helmlinger D, Hardy S, Ahou-Slyeman G, Eberlin A, Bowman AB, Ganssmulder A, Picaud S, Zoghbi HY, Trottier Y, Tora L, Devys D. 2006. Glutamine-expanded ataxin-7 alters TFF1/TFF2 STAGA recruitment and chromatin structure leading to photo-receptor dysfunction. PLoS Biol 4:e67.

Jarmazhi E, Kiefer J, Savas S, Briollais L, Tuzemen S, Pabalan N, Ibrahim-Zada I, Mousses S, Ozcelik H. 2008. Discovery of genetic profiles impacting response to chemotherapy: Application to gemcitabine. Hum Mutat 29:461–467.

Karras JR, Puisais GA, Huechner K. 2014. Replicative Stress and the FHHT Gene: Roles in tumor suppression, genome stability and prevention of carcinogenesis. Cancers (Basel) 6:1208–1219.

Keller W, Minvielle-Sebastia L. 1997. A comparison of mammalian and yeast pre-mRNA 3'-end processing. Curr Opin Cell Biol 9:329–336.

Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, Sakamoto H, Tsuta K, Furuta K, Shimada Y, Iwakawa R, Ogawa H, Oike T, Enari M, Schetter AJ, Okayama H, Haugen A, Skaug V, Chiku S, Tamanaka I, Arat Y, Watanabe S, Sekane I, Ogawa S, Hanzawa T, Tsuda H, Yamasaki T, Shibuta T. 2012. KIF5B-RE7 fusions in lung adenocarcinoma. Nat Med 18:375–377.

Kondo A, Hashimoto S, Yano H, Nagayama K, Mazaki Y, Sabe H. 2000. A new resiniferatoxin-binding protein, PAG3/Papulapa/XIAA0400, bearing an ADP-ribosylation factor GTAPase-activating protein activity, is involved in pinaxillin recruitment to focal adhesions and cell migration. Mol Cell Biol 21:1515–1527.

Lamesch P, Li N, Milstejn S, Fan C, Hao T, Naka H, Hu Z, Venkatesan K, Bethel G, Martin P, Rogers J, Lawlor S, McLaren S, Drictor A, Bonic H, Cusick M, Vandenbaste H, Dunham J, Hill DE, Vidal M. 2007. hORFeome v3.1: A resource of human open reading frames representing over 10,000 human genes. Genomics 89:307–315.

Li A, Liu Z, Lezon-Geyda K, Sarkar S, Lannin D, Schulz V, Krop I, Elner K, Harris L, Tuck D. 2011. GPHMM: An integrated hidden Markov model for identification of copy number alterations and loss of heterozygosity in complex tumor samples using whole genome SNP arrays. Nucleic Acids Res 39:4928–4941.

Li H, Wang J, Mor G, Sklar J. 2008. A neoplastic gene fusion mimics trans-splicing of RNAs in normal human cells. Science 321:1357–1361.

Liu Q, Zhao XY, Bai RZ, Liang SF, Nie CL, Yuan Z, Wang GT, Wu Y, Chen LJ, Wei YQ. 2009. Induction of tumor inhibition and apoptosis by a candidate tumor suppressor gene DRR1 on chromosome 9p. Cancer Res 69:1824–1833.

Loewy AD, Niles KM, Anastasio N, Watkins D, Lavoie J, Lerner-Andresen J, Picaud S, Zoghbi HY, Trottier Y, Tora L, Dunham I, Hill DE, Vidal M. 2007. hORFeome v3.1: A resource of human open reading frames representing over 10,000 human genes. Genomics 89:307–315.

Matsui C, Kaieda S, Ikegami T, Mimori-Kiyosue Y. 2008. Identification and characterization of a link between the SAMP repeats of adenomatous polyposis coli tumor suppressor and the Src homology 3 domain containing a novel dependence. Mol Genet Metab 96:261–267.

Mehta R, Liu S, Naka H, Hu Z, Venkatesan K, Bethel G, Martin P, Rogers J, Lawlor S, McLaren S, Drictor A, Bonic H, Cusick M, Vandenbaste H, Dunham J, Hill DE, Vidal M. 2007. hORFeome v3.1: A resource of human open reading frames representing over 10,000 human genes. Genomics 89:307–315.

Ninomiya K, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, Sakamoto H, Tsuta K, Furuta K, Shimada Y, Iwakawa R, Ogawa H, Oike T, Enari M, Schetter AJ, Okayama H, Haugen A, Skaug V, Chiku S, Tamanaka I, Arat Y, Watanabe S, Sekane I, Ogawa S, Hanzawa T, Tsuda H, Yamasaki T, Shibuwa T. 2012. KIF5B-RE7 fusions in lung adenocarcinoma. Nat Med 18:375–377.

Pinto MT, Sousa S, Corso G, Lin ML, McBride DJ, Maddison M, Magure S, McIay K, Genez A, Valcarcel J, Mullerd L, Mudic L, O'Meara S, Pleasance E, Rajasingham A, Shepherd R, Smith R, Stebbings L, Stephens P, Tang G, Tarpey PS, Turrell K, Dykema KJ, Khoo SH, Sim D, Wondergem B, Anema J, Kuhnsiozki RJ, Teh BT, Stratton MR, Futrela PA, 2010. Systematic sequencing of renal carcinoma reveals inheritance of histone-modifying genes. Nature 463:360–363.

Pinto MT, Liu S, Naka H, Hu Z, Venkatesan K, Bethel G, Martin P, Rogers J, Lawlor S, McLaren S, Drictor A, Bonic H, Cusick M, Vandenbaste H, Dunham J, Hill DE, Vidal M. 2007. hORFeome v3.1: A resource of human open reading frames representing over 10,000 human genes. Genomics 89:307–315.

Poon C, Hsu JY, Liu Z, Wu Y, Chen LJ, Wei YQ. 2009. Induction of tumor inhibition and apoptosis by a candidate tumor suppressor gene DRR1 on chromosome 9p. Cancer Res 69:1824–1833.
fusion discovery in tumor RNA-Seq data. PLoS Comput Biol 7: e1001138.

Milne RL, Burwinkel B, Michailidou K, Arias-Perez J, Zamora Pharoah PD, Hall P, Giles GG, Baglietto L, Haiman CA, Henderson BE, Schumacher F, Wang X, Olson JE, Vachon C, Purrington K, Severi G, Toland AE; TNBCC, Yannoukakos D, Sangrajrang S, McKay J, Hartikainen JM, Shu XO, Lu W, Gao YT, Couch FJ, Siriwanarangsan P, Mannermaa A, Kataja V, Kosma VM, Wu AH, Tseng CC, Van Den Berg D, Stram DO, Bogdanova Flesch-Janys D, Winqvist R, Pylkäs K, Santos-Silva I, Peto J, Zheng W, Deming-Halverson S, Ouweland AM, Koppert LB, Fletcher O, Johnson N, D Nordestgaard BG, Flyger H, Hooning MJ, Kriege M, van den Endewaard BG, Varmahlismaki E, Zhang D, Wang X, Zhao J, Prazeres H, Julião S, Nogueira Neto T, Muir K, Lophatananon A, Stewart-Brown S, De Angelis K, Pita G, Alonso MR, Dennis J, Wang Q, He F, Wong SC, Ong CN, Shah M, Molenaar PM, Menzies A, Mudie L, Pritchard CC, Wong CA, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H. 2015. Identification of EMILIN-1/ALK fusion gene in non-small-cell lung cancer. Nature 448:561–566.

Sugawara E, Togashi Y, Kuroda N, Sakata S, Hatanaka K, Sato Y, Loo J, Arias-Perez J, Kitagawa M, Mano H, Ishikawa Y, Takeuchi K. 2012. Identification of anaplastic lymphoma kinase fusions in renal cancer: Large-scale immunohistochemical screening by the intercalated antibody-enhanced polymer method. Cancer 118:4427–4436.

Takeuchi K, Soda M, Togashi Y, Suzuki R, Nakamura H, Hatanaka S, Sato Y, Hatanaka W, Minomiya H, Uehara H, Lim Choi Y, Sato Y, Okumura S, Nakagawa K, Mano H, Ishikawa Y. 2012. RET, ROS1 and ALK fusions in lung cancer. Nat Med 18:328–381.

Tanuma N, Nomura M, Ikeda M, Kasuga T, Tsubuki Y, Takagaki K, Kawamura T, Yamashita Y, Sato I, Sato M, Katakura R, Kikuchi K, Shimizu H. 2009. Protein phosphatase Dusp26 associates with RIF1 motor and promotes N-cadherin-mediated cell-cell adhesion. Oncogene 28:752–761.

The Cancer Genome Atlas Research Network. 2013. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 494:43-49.