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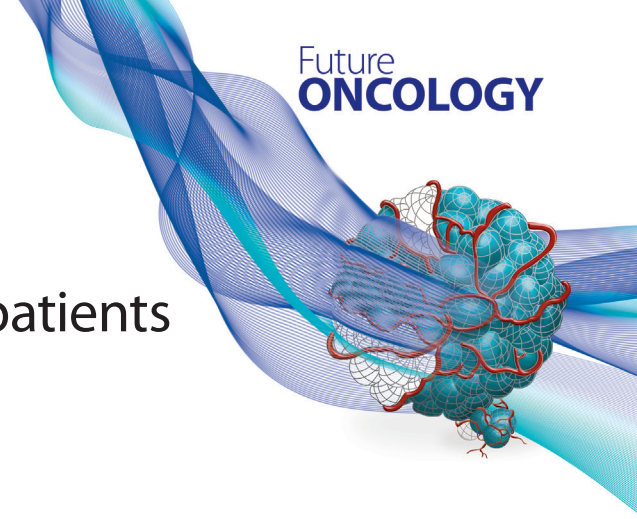

Clinical significance of *NDRG3* in patients with breast cancer

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Aim: The expression level of *NDRG3* gene is investigated among breast cancer (BC) patients. **Methods:** Real-time quantitative PCR was performed. **Results:** *NDRG3* was downregulated in BC patients particularly in advanced stage of the disease. HER2 status was significantly correlated with the expression of *NDRG3*. Also, triple-negative BC patients showed low levels of *NDRG3* expression in comparison to other subtypes. Lastly, the expression of *NDRG3* had significant impact on survival, with *NDRG3* downregulated patients having the worst event-free survival rate among others. **Conclusion:** We have presented that NDRG3 might be a tumor suppressor candidate. NDRG3 downregulation might be involved in the tumorigenesis and development of invasive BC in an advanced phase of the disease.

First draft submitted: 12 October 2016; Accepted for publication: 20 January 2017; Published online: 27 February 2017

KEYWORDS
• breast cancer • MYC
• NDRG

Breast cancer (BC), the most prevalent cancer among women, is a very heterogeneous disease. In its pathological features, some instances of BC show slow growth with good prognosis, whereas others are aggressive and invasive [1]. In spite of noticeable improvements in early diagnosis and development of novel therapeutic strategies, BC remains with a large number of deaths ascribed to recurrent or metastatic events [2,3]. Hence, it is essential to specify molecular players which may expand our understanding of this disease, and help in the identification of new targets for treatment and diagnostic indicators [4].

MYC is one of the key yet discovered molecules in human cancer biology. Originally identified as an onco-protein, Myc is involved in numerous biological processes, such as cell proliferation, differentiation, apoptosis, via transcriptional regulation of its target genes [5]. High amplification and overexpression of *MYC* genes in various types of human cancers have been reported, previously [6,7]. Among Myc target genes, the human N-myc downstream-regulated gene (NDRG) family, has been a focus of research in recent years [8].

NDRG family is composed of four members: NDRG1, NDRG2, NDRG3 and NDRG4, that the degree of amino acid homology between each other is 53–65% [9]. Functional studies have revealed that NDRG family plays vital roles in carcinogenesis and may be used as biomarker in numerous types of cancers. Up/downregulation, as well as oncogenic or tumor suppressor functions influencing key hallmarks of tumorigenesis such as proliferation, invasion, differentiation and migration, have been reported. NDRG1 and NDRG2 have been thoroughly studied, whereas

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limited information is available for NDRG3 and NDRG4, concerning their pattern of regulation and function [10]. Although the function of NDRG3 is yet unknown, genomic studies have suggested that it could be involved in regulation of a large number of genes associated with cancer progression [8,11].

Human *NDRG3* is located on chromosome 20q11.21–11.23. It is 2588 base pairs in length, encoding a 363 amino acid polypeptide (40 kD) that is highly conserved compared with mouse ndr3 protein. NDRG3 is upregulated in a wide range of tissues including ovary, prostate, testis, brain, spinal cord, thymus rudiment, heart and kidney [12]. In the present study we investigated the expression of *NDRG3* in tumor tissues of BC patients with different subtypes and stages in comparison to that of normal breast tissues. We suggest that it might be functioning as a tumor suppressor in BC, as advanced stage and triple-negative patients showed the lowest expression of *NDRG3* among others. Also, we find that the group of patients with *NDRG3* upregulation have better survival rate compared with medium or low expressing groups.

Methods

• Study population

In the present case–control study, 88 tumors of breast and six normal breast tissue samples, from healthy individuals with no family history of malignancy, were obtained from the Breast Cancer Research Center Biobank [13]. All patients were clearly identified as having BC based on the clinicopathological and histological criteria. According to the patients' history, none of them had received preoperative chemotherapy and/or radiotherapy, and had no other malignancies. The contributed demographic, histopathology and comprehensive clinical profile of enrolled individuals for surgery who agreed to donate their breast samples, were transferred and recorded in coordination with their clinical reports. Also, the status of estrogen receptor (ER), progesterone receptor (PR) and HER2 was determined. Details regarding the sample preparation information are previously described [13].

• Quantitative real-time PCR

According to the protocols followed by Breast Cancer Research Center Biobank, after excisional biopsy or surgery performed, the content of cancer cells in each sample was pathologically checked and immediately sample tissues were snap-frozen in liquid nitrogen and stored at -70°C. RNA extraction and cDNA synthesis were done as previously explained [14].

SYBR Green quantitative real-time PCR was performed for *NDRG3* and *HPRT* genes **(Table 1)**, where the latter was chosen as housekeeping gene, using TAKARA kit (Shiga, Japan) based on manufacturer's protocol in duplication for each gene. Primers for above-mentioned genes are listed in **Table 1**. Samples were amplified in ABI7500 PCR machine (Applied Biosystems, CA, USA). Thermal cycling conditions included an initial denaturation step (Reps 1) at 95°C for 30 s, followed by step 2 (Reps 40) at 95°C for 5 s and 34 s at 60°C.

Analysis of the results was done using REST 2009 software. The relative gene expression was estimated normalizing to normal breast tissue controls. Among three used housekeeping genes in breast tissue, *HPRT* showed the least variation in gene expression between our normal and tumor samples [15].

Each reaction was followed by melting curve analysis involving heating of the PCR product from 60 to 95°C. The curve in the specimen was compared with the curve of positive control to recognize specific amplicon from primer dimer formation. The melt point of 83°C was defined for *NDRG3* amplicon. Reactions with this melting point were considered positive for *NDRG3* expression. Any amplicon with other melt point was regarded as nonspecific amplification. Data were analyzed using SDS software, vers.2.0 (Applied Biosystems). Also, relative quantity (RQ) of the patient's tumor samples was calculated using ΔΔCT method. RQ differences greater than 2 and less than 1 are considered as upregulation and downregulation, respectively.

• Statistical analysis

Statistical analyses were performed using SPSS software (version 18). Descriptive statistical analyses and non-parametric tests, including Mann–Whitney U and Kruskal–Wallis tests were used. Also, Chi-Square and Fisher's Exact Tests were performed for analysis of qualitative variables. For all analyses *p*-value was measured and $p < 0.05$ was considered as significant. The Kaplan–Meier method was used to estimate event-free survival rates and comparison of the survival between groups was performed with the log–rank test (Mantel–Cox). Event-free survival was calculated from the day of sample collection until death or last follow-up, regardless of the

Table 1. Primers used for real-time quantitative PCR.

Gene	Sequence	Length (mer)	Tm (Ċ)
HPRT (forward)	CTGGCGTCGTGATTAGTGATG	21	55.3
HPRT (reverse)	CGTTCAGTCCTGTCCATAATTAG	23	52.2
NDRG3 (forward)	TGGAGCTTACATCCTCAGCAG	21	54.3
NDRG3 (reverse)	CCAGTCAATCCAGCCTTTAGC	21	55.2

cause of death. Additionally, the multivariable Cox proportional hazards regression model was used to determine multivariate hazard ratios (HRs) for hormone receptor variables and event-free survival.

Results

The mean age of patients at the time of diagnosis was 49.97 years (±10.72; range: 24–78 years). 11.4% of them were diagnosed in grade 1, 58% in grade 2 and 21.6% in grade 3. Out of 88 enrolled patients, 34.1% of them were ER negative, 62.5% ER positive, 38.6% PR negative and 54.5% PR positive, while 71.6% of them were HER2 negative and 25% HER2 positive.

The median RQ of *NDRG3* gene expression level in BC patients is 0.62. In the case group, 49 (55.7%) samples showed downregulation, nine (10.2%) showed normal expression and 30 (34.1%) showed upregulation of *NDRG3*. A total of 62.5% of the patients harboring stage I tumor showed downregulation and patients with stage II and III demonstrated *NDRG3* downregulation with the respect percentages of 51.3 and 60%. Finally, stage IV patients showed significantly the least mRNA expression level of *NDRG3* ($p = 0.000$). The correlation between patients' clinicopathological characteristics and the mRNA levels of *NDRG3* in tumor samples is demonstrated in **Table 2**. Although ER/PR-negative patients showed lower *NDRG3* expression in comparison to other ER/PR status patients **(Figure 1A)**, the difference between the expression of *NDRG3* in ER/PR-negative with ER/PR-positive and other ER/PR status patients were not statistically significant ($p = 0.179$; $p = 0.328$). Triple-negative ($ER^-/PR^-/HER2^-$) patients demonstrated significant *NDRG3* downregulation in comparison to other ER/PR/HER2 status patients which is shown in **Figure 1B** ($p = 0.033$). We further sought to investigate whether there is any correlation between *NDRG3* expression levels and certain subtypes of BC. In the group of patients with *NDRG3* downregulation, significant correlation was found between the PR status and *NDRG3* mRNA expression level ($p = 0.037$). Also, in the group of patients with *NDRG3* upregulation, there was a correlation with the ER status ($p = 0.033$).

Moreover, we also investigated the correlation of patients' survival rate with *NDRG3* expression levels. Kaplan–Meier survival analysis revealed significant difference between the median survival durations of BC patients with down/normal/upregulation of *NDRG3*, with downregulated patients having the worst event-free survival ($p = 0.036$, log–rank test; **Figure 2**). Cox proportional hazard modeling showed that female sex hormone receptors, ER (HR: 0.74; $p = 0.62$) and PR (HR: 1.28; $p = 0.67$), were not risk factor for survival.

Discussion

BC is one of the most heterogeneous diseases with several subtypes, each with unique characteristics, oncogenic drivers or pattern of tumor suppressor loss. In this study, we investigated the expression pattern of *NDRG3* among different stages and subtypes of BC patients. The results showed that more than half of the patients express downregulation of *NDRG3*, with stage IV tumors showing the lowest expression levels. In addition, there was a significant correlation between the expression of *NDRG3* and HER2 status, and in patients with its downregulation, significant correlation was found between the PR status and *NDRG3* expression level. However, in patients with NDRG3 upregulation, there was a correlation between the ER status and *NDRG3* expression. Also, our results indicated that the difference between the expression of *NDRG3* among triple-negative patients compared with other subtypes was significant. Furthermore, we show that downregulation of *NDRG3* expression coincides with reduced event-free survival.

The NDRG gene family consists of four members, NDRG1, NDRG2 and two novel members, NDRG3 and NDRG4. Phylogenic analysis demonstrated that human NDRG1 and NDRG3 belong to one subfamily, whereas NDRG2 and

Table 2. *NDRG3* gene expression and clinicopathological factors in breast cancer patients.

Clinicopathological variables	Median RQ†	Specified p-value (within groups)	p-value (between groups)
Age (years):			0.65
– <50	0.32	0.56	
– >50	0.37	0.06	
Stage:			0.49
– I	0.60	0.60	
– II	0.64	0.97	
– III	0.52	0.40	
– IV	0.26	0.00	
Grade:			0.78
– 1	1.02	0.89	
– 2	0.61	0.85	
– 3	0.40	0.10	
ER:			0.33
– +	0.61	0.63	
– -	0.57	0.97	
PR:			0.13
– +	0.63	0.60	
– -	0.60	0.69	
HER2:			0.01
– +	0.66	0.15	
– -	0.61	0.97	

†Median RQ level of *NDRG3* mRNA expression in breast cancer patients.
RQ: relative quantity.

NDRG4 belong to another [9]. Although the roles of NDRG members has not been fully understood, it can be inferred from the previous studies that these proteins have important functions in tumor development and differentiation [10]. It was shown that NDRG1 is downregulated in BC cells, especially in patients with advanced stages. Notably, *in vitro* analyses showed that upregulation of NDRG1 suppressed invasive properties of cells [16]. Bandyopadhyay *et al.*, investigated the expression of NDRG1 in 85 BC cases, and found no correlation between its expression and histological grade and age. Their study revealed that NDRG1 had a significant adverse correlation with metastatic spread to bone and lymph node [16]. Mao *et al.*, reported that NDRG1 expression did not correlate with tumor size, age and lymph node metastasis, however, it was correlated with tumor stage in invasive BC [17]. On the other hand, the expression of NDRG2 is found to be downregulated in cancer patients, and its upregulation shown to negatively regulate proliferation, metastasis, migration and invasion of cancer cells [18,19]. However, it has been stated by Wei *et al.* that it is not totally clear whether NDRG2 downregulation is a cause or a consequence of the tumor progression from normal tissue [18].

The comparison between the expression of *NDRG3* in normal and tumor tissues, and also the negative correlations of its expression levels and the stages of BC suggest that the loss of *NDRG3* expression might take part in the carcinogenesis and progression of invasive BC. This notion supports the tumor-suppressive function of NDRG3. In contrast, Wang *et al.* found the upregulation of *NDRG3* in 58.6% of prostate cancer specimens, while it was upregulated only in 13.2% of benign prostatic hyperplasia samples. Also, it was shown *in vitro* that *NDRG3* upregulation coincides with an increase in growth rate, colony formation and migration of prostatic human carcinoma cells, while its knock-down suppresses the growth rate and the colony-forming abilities of the cells. Moreover, *in vivo* analyses show that *NDRG3* upregulation increases tumor growth in nude mice, pointing out an oncogenic mode of action [11]. They studied the expression pattern of NDRG3 among several prostatic cancer cell lines, through which suggesting that androgen-independent mechanisms might be involved in the regulation of its. It pointed out the concept that depending on its expression in androgen-dependent or androgen-independent cells, NDRG3 might function differently [11]. In another study, significant

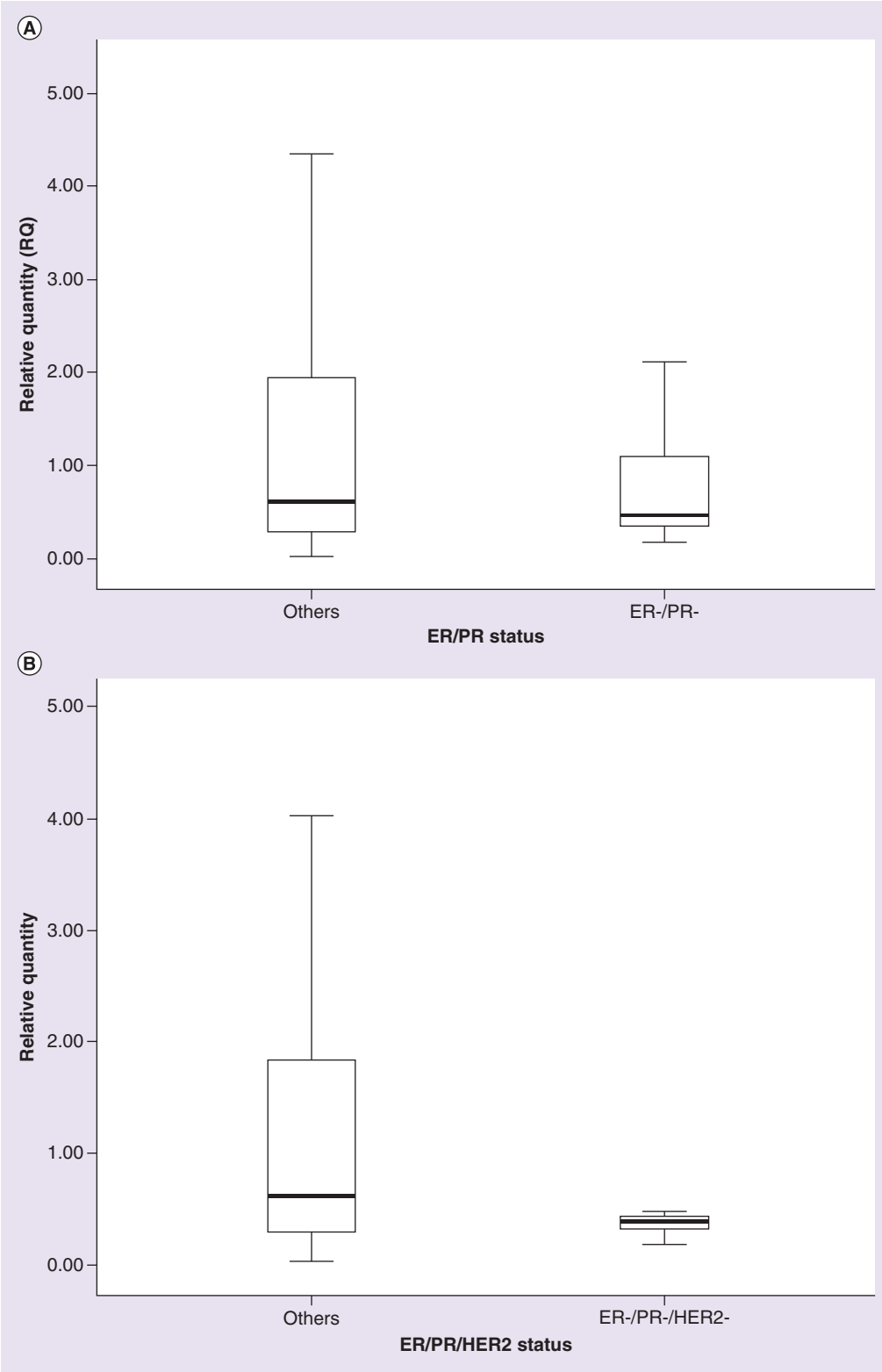

Figure 1. The comparison of *NDRG3* expression in different groups of breast cancer patients.
(A) *NDRG3* mRNA expression in tumor tissue of BC patients with ER-/PR- and the other ER/PR status.
(B) *NDRG3* mRNA expression in tumor tissue of BC patients with ER-/PR-/HER2- and the other ER/PR/HER2 status.

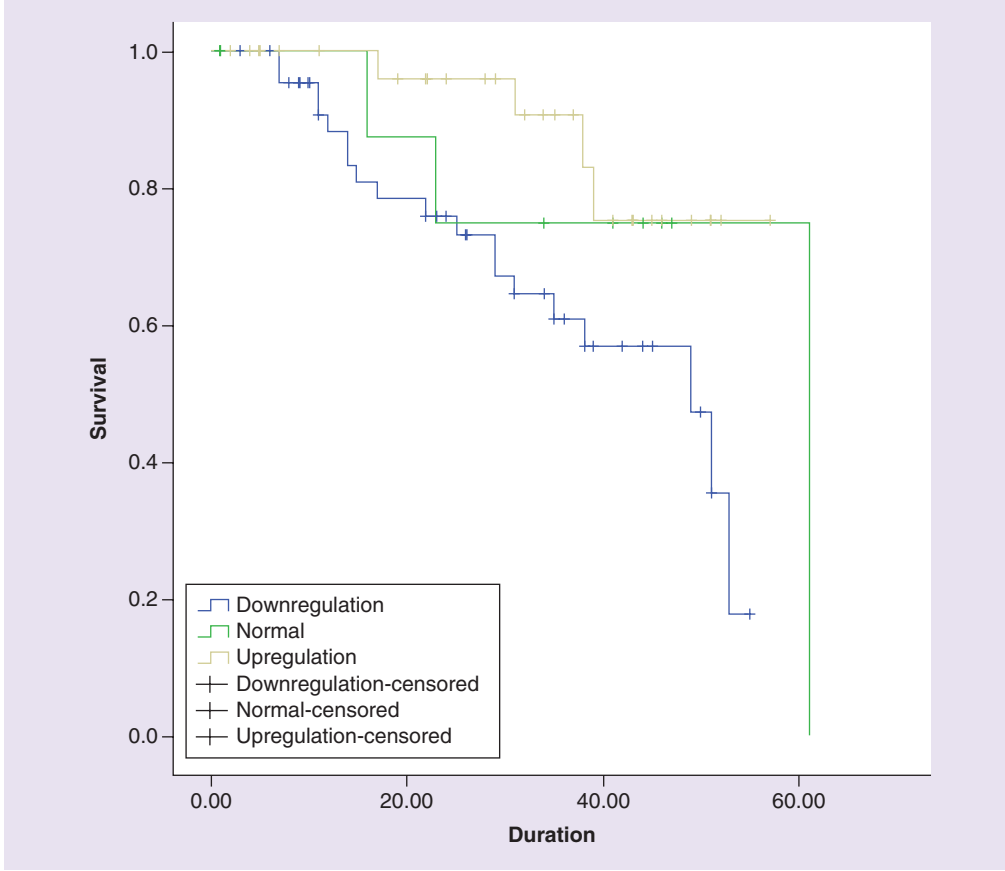

Figure 2. Kaplan–Meier survival analysis of the breast cancer patients according to the NDRG3 mRNA expression. mRNA expression levels were normalized to the amount of HPRT, and were divided into down/normal/upregulation groups based on their relative expression levels.

correlation between *NDRG3* upregulation with advanced disease stage and metastatic event was found [20]. NDRG1, which is classified in the same subfamily as NDRG3, shows similar regulation pattern compared with NDRG3 in BC [21]. Review of the previous studies reveals that there is a controversy regarding the function of NDRG family members. These paradoxical results concerning NDRG3 expression point out that this protein may have tissue-specific functions in different cancer types. Furthermore, several other factors such as chemicals and hormones that induce carcinogenesis, the level of hypoxia, and genetic or epigenetic modifications in oncogenes and tumor suppressor genes affect the regulation of NDRG3. These effectors may modify the outcome of the NDRG3 regulation in various types of cancers, as they influence other NDRGs members [22–25]. Overall, it seems that tumor suppressing mode of action is more concordant with NDRGs function, and this property is more consistent with other tumor suppressor genes than oncogenes. Considering this point, expression of all NDRG family members seem to be associated with progressive stages of differentiation, increasing from birth to adulthood. Although low levels of expression may be detected from a relatively early embryonic stage, expression always increases in the postnatal and mature animal [10,26].

Fan *et al.* reported an inverse correlation between the expression of miR-122 and NDRG3. They showed that downregulation of miR-122 leads to upregulation of NDRG3, which induces tumorigenesis in hepatocellular carcinoma [27]. Interestingly, miR-122 was reported to be upregulated in BC, particularly in advanced stage of tumors [28]. This was shown to facilitate tumor progression through reprogramming systemic energy metabolism [29]. Hence, it seems that downregulation of NDRG3 can be expected in breast tumor tissues in comparison to normal breast tissues, likely as a result of reverse regulation by miR-122. This regulatory mechanism is

further of note, as Wu *et al.* found strong correlation between miR-122 and HER2 status [28], as significant correlation between NDRG3 and HER2 status was seen in the present study.

Association between hormone receptors as molecular markers with *NDRG3* expression suggests the importance of this gene for further investigation as a therapeutic target. NDRG3 expression might be influenced by the molecular cascades triggered by these receptors. However, there has been few studies investigating this notion for the NDRG family. NDRG3 has been identified as activated by the ER-β and as a component of an antiproliferative response induced by ER-β in the T47D BC cell line [11,30]. Bandyopathyay *et al.*, showed that there was not a significant correlation between NDRG1 expression and ER status in BC [21]. However, previous studies reported that NDRG1 expression is influenced by androgens in prostate cancer cells [31,32]. Immunoreactivity of NDRG1 was observed in glandular epithelia of normal lactating breast tissue by Lachat *et al.* [33]. NDRG1 expression may greatly differ according to the absence or presence of hormonal pathways in hormone-susceptible tumors such as breast and prostate tumors. In addition, studies in BC revealed that NDRG1 is a molecular indicator of the therapeutic efficacy of anti-estrogenic agents [34,35]. We suggest that other molecular markers and epigenetic alterations must be evaluated besides NDRG3 expression. For example Wei *et al.* [18] showed that epigenetic alterations including methylation or histone deacetylase during tumorigenesis lead to NDRG2 expression deregulation, a process in which no mutations in the coding region of NDRG2 is involved.

Conclusion

We have presented that NDRG3 could be a tumor suppressor candidate, suggesting that downregulation of NDRG3 may play an important role in development of cancer and undesirable outcomes. However, the precise role of NDRG3 in BC has not been clarified and the functional consequences of its downregulation remained to be investigated. In the continuation of this work, we will evaluate the protein expression of NDRG3. It seems that the different oncogenic- or tumor-suppressive functions of NDRG3 occurs in different tumor types. Gene therapy utilizing systems to overcome the loss of tumor suppressor genes is being studied as a novel treatment modality with the expectation of improving therapeutic efficacy [36]. Hence, understanding the role and function of NDRG3 in BC may help to establish a new target for such strategies. Even though, we found correlations between NDRG3 and hormone receptor markers, the molecular mechanisms and signal transduction pathways that are involved in these processes are still unknown. In this regard, genome-wide investigation of signal transduction pathway components is suggested.

Acknowledgements

The authors would like to thank all patients who contributed to the present study.

Financial & competing interests disclosure

This work was funded by the Breast Cancer Research Centre, ACECR, Iran. The authors have no other relevant affiliations or financial involvement with any organization

EXECUTIVE SUMMARY

- NDRG1 and NDRG2 have been thoroughly studied, whereas limited information is available about two novel members, NDRG3 and NDRG4, concerning their pattern of regulation and function.
- *NDRG3* was downregulated in breast cancer (BC) patients particularly in advanced stage of the disease.
- HER2 status was significantly correlated with the expression of NDRG3 ($p = 0.01$).
- Triple-negative BC patients showed low levels of *NDRG3* expression in comparison to other BC patient groups ($p = 0.033$).
- The expression of *NDRG3* had significant impact on survival, with downregulated *NDRG3* patients having the worst event-free survival rate among others ($p = 0.036$).
- Present study presented that NDRG3 might be a tumor suppressor candidate, and it might have tissue-specific functions in different cancer types.
- This study has revealed substantial relationships between one of the NDRG members with hormone receptors, suggesting new target in the challenge of resistant BC cells.

or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

BCRC-BB is obliged to ethical guidelines and recommendations for biobanks on the storage and use of human biological samples. The study was approved by the BCRC Ethics Committee which is in compliance with the Helsinki declaration. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Targeting *EGFR* mutation in non-small-cell lung cancer: challenges and future perspectives



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First draft submitted: 11 June 2016; Accepted for publication: 22 August 2016; Published online: 14 September 2016

Lung cancer represents the leading cause of cancer death worldwide [1]. At diagnosis, the majority of patients present an advanced or metastatic disease; despite the improvement in diagnostic techniques and therapeutic strategies, lung cancer prognosis remains poor with less than 15% of patients surviving more than 5 years [2]. In non-small-cell lung cancer (NSCLC), advanced disease (stage IIIB and IV) is treated by platinum-based regimens: cisplatin or carboplatin in combination with gemcitabine, vinorelbine, taxanes (docetaxel or paclitaxel) or pemetrexed. These different regimens showed similar efficacy in terms of overall survival (OS) with a median survival rate of 8–12 months, despite different toxicity profiles [3]. In the specific case of adenocarcinoma, the combination pemetrexed/cisplatin was shown to be more effective than gemcitabine/cisplatin with a superior OS (12.6 vs 10.9 months) and a better tolerability profile, and is therefore considered the most used standard in this population [4].

A new era of lung cancer treatment began with the discovery of multiple driving mutations, mainly within adenocarcinoma samples. These mutations occur most commonly in oncogenes. However, few mutations so far resulted in therapeutic implications such as mutant *EGFR*, fusion of the *ALK* gene, *ROS1* or *RET*. Other mutations of potential interest, *BRAF* and *ERBB2*, are still under investigation. Frequent mutations were also identified, as within the *KRAS* gene, but are of no added therapeutic benefit and are treated with conventional chemotherapy. Abnormalities were also detected within tumor suppressor genes such as *TP53*, *STK11*, *CDKN2A8*, *KEAP1* and *SMARCA4*; however, no targeted therapy is available at the moment aiming these genes [5]. More recently, and with the tsunami of immune checkpoint inhibitors, anti-PD1 agents become an interesting weapon in the armamentarium of lung cancer treatment independently of the mutational status [6].

KEYWORDS
• *EGFR* • non-small-cell lung cancer
• T790M

"At diagnosis, the majority of patients present an advanced or metastatic disease; despite the improvement in diagnostic techniques and therapeutic strategies, lung cancer prognosis remains poor with less than 15% of patients surviving more than 5 years."

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"*EGFR* testing has become a standard in non-small-cell lung cancer treatment. Every patient presenting with a sensitizing *EGFR* mutation should be treated by a first- or second-generation tyrosine kinase inhibitor."

The *EGFR* mutation was the first mutation to be targeted by novel agents, the tyrosine kinase inhibitors (TKIs). Its occurrence varies by ethnicity; ranging from around 16% in Caucasian populations to more than 50% in selected Asian populations [7,8]. Yet these mutations are not equivalent; they tend to occur between the regions 18 and 21 of the receptor. The most frequent are exon 19 deletion mutations and the single-point substitution mutation L858R in exon 21, both conferring sensitivity to TKI. On the other hand, tumors may subsequently develop resistance to previously effective TKI therapy due to an emergent mutation in exon 20 of the *EGFR* gene where a methionine is substituted for threonine at position 790 (T790M), as described for the first time in 2005 [9].

The first molecule evaluated in the first-line setting of lung nonsquamous adenocarcinoma with *EGFR* mutation, was gefitinib in 2009. When compared with carboplatin/paclitaxel in the IPASS trial, gefitinib showed noninferiority with respect to progression-free survival (PFS; 5.7 vs 5.8 months). On the other hand, no difference in OS was noted in both arms, probably due to the crossover effect (18.8 vs 17.4 months) [8]. This trial led to the approval of gefitinib in the first-line setting for advanced NSCLC-harboring *EGFR* mutation, which occurs mostly in women who never smoke, of Asian origin and having adenocarcinoma as a histologic subtype.

Another drug, erlotinib, was also tested in the advanced setting of NSCLC. The EURTAC trial published in 2012 compared the safety and efficacy of first-line erlotinib against platinum-based chemotherapy in non-Asian patients with advanced *EGFR*-mutatnt NSCLC. PFS was significantly better in the erlotinib arm compared with standard chemotherapy (9.7 vs 5.2 months) with a hazard ratio (HR) of 0.37, and $p < 0.0001$. Overall response rate was higher in patients treated with erlotinib, 64%, compared with those treated with chemotherapy, 18%; nevertheless OS did not differ significantly between the two groups. On the basis of these results, the drug was approved as first-line treatment for advanced *EGFR*-mutant NSCLC [10].

After the positive results achieved in the treatment of advanced *EGFR*-mutant NSCLC with first-generation TKIs, second-generation molecules were tested. The latter are more potent, targeting not only the EGFR, but also other members of the ErbB family. Afatinib, a second-generation TKI, was tested in two sister trials, the LUX-Lung 3 and LUX-Lung 6 in comparison with chemotherapy and was found to be significantly superior in terms of PFS in both studies, with a median PFS of 11.1 versus 6.9, and 11.0 versus 5.6, respectively [11,12]. Subsequently, afatinib was approved in 2013, as a first-line treatment for NSCLC whose tumors have *EGFR* exon 19 deletions or exon 21 (L858R) substitution mutations as detected by a US FDA approved test. Regarding OS, a combined analysis of both studies showed a significant difference between the afatinib and the chemotherapy arms with respect to the common mutations (27.3 vs 24.3 months) and to the exon 19 deletion subgroup (31.7 vs 20.7 months) with an HR of 0.59; whereas no difference in OS was noted in the L858R population. LUX-Lung 7 compared afatinib and gefitinib head to head, and showed a significant difference in terms of PFS favoring afatinib (11.0 vs 10.9 months) with an HR of 0.73 (95% CI: 0.57–0.95). Regarding side effects, grades 3 or worse adverse events (AEs) were mainly diarrhea, rash, acne and fatigue in the afatinib arm; and mostly AST/ALT derangements and rash or acne in the gefitinib arm [13]. In addition, all three drugs demonstrated similar outcomes in terms of quality of life. In fact, the above studies showed that quality of life was better when using TKI compared with chemotherapy.

Despite the benefit obtained from the above agents with tumor harboring an *EGFR*-sensitizing mutation (exon 19 deletion or L858R substitution in exon 21), with a controlled disease for around 1 year; patients will progress. The most commonly described mechanism of resistance to TKI, in more than half of the cases, is the presence of *de novo* T790M mutation [9]. In order to allow for disease control again, new agents were needed to overcome resistance mechanisms.

Osimertinib is a third-generation TKI tackling the T790M mutation and enabling disease control in previously treated, progressing *EGFR*-mutant patients as seen in the AURA 1 and AURA 2 trials with a median PFS of 8.6 months in the second-, third- or fourth-line settings, and a disease control rate of 92%. However, no benefit was seen when using osimertinib in the T790M-negative population with a PFS of 2.8 months. Based on these two studies, osimertinib was approved by FDA and EMA for pretreated T790M mutation-positive patients [14,15].

Furthermore, and unlike other EGFR-TKI, osimertinib is able to cross blood–brain barrier and is therefore effective in preventing and treating brain metastasis. In fact, the results of BLOOM Phase I trial presented at the ASCO 2016 annual meeting supported these data by showing that osimertinib reduced CNS lesions independently of the presence of the T790M mutation. The drug was tolerated with manageable toxicity profile; with most AEs being diarrhea most of which was of grades 1 and 2, nausea and rash of grades 1 and 2. No hyperglycemia or interstitial lung disease was noted [16].

After proving its efficacy in overcoming the T790M mutation, osimertinib was tested in the first-line setting in *EGFR*-mutant T790m-negative patients. The overall response rate was 77%. Median PFS has not been reached yet for the 80 mg dose, but is superior to the 1-year PFS of first- and second-generation TKIs. The drug showed an acceptable toxicity with few AEs at the dose of 80 mg daily. It is important to note that the patients who progressed after an initial treatment by osimertinib did not have the T790M mutation as mechanism of resistance. These results indicate that the use of osimertinib in the first-line setting could change the biology of the disease [17].

Overcoming the T790M mutation was also the target of olmutinib, a novel third generation EGFR-TKI that showed promising results when data were presented at ASCO 2016 with 54% of patients presenting an objective response with a median duration of 8.3 months. In addition, side effects related to the use of olmutinib were mild to moderate and consisting mainly of diarrhea, rash and nausea, similar to those seen with other EGFR-TKI [18]. More results for this drug are awaited with the ELUXA Phase I trial and other Phase II trials.

Another third-generation TKI under development is rociletinib which was trialed in the TIGER-X Phase I study. It showed early efficacy in T790M-positive individuals, a disease control rate of 93% and PFS of 8 months [19]. However, FDA approval was not granted and therefore further development of this molecule was interrupted. In patients treated with rociletinib, *EGFR* mutation detection in circulating tumor DNA from blood and urine was compared with that in matched tissue. The results were presented at the 2016 ASCO annual meeting and showed a similar proportion of detecting the T790M mutation in *EGFR*-mutant patients, around 80%, in tested tissues, urine and plasma by genotyping. Moreover, urine and plasma tests are not considered an alternative to tissue sampling rather than a complement to solid biopsy which can detect T790M mutations missed by this latter approach probably due to tumor heterogeneity of inadequate sample quality. As for response rates, T790M-positive patients presented a similar objective response rate, PFS and duration of response to TKI treatment independently of biopsy method whether it was a tissue biopsy or a liquid biopsy from urine or plasma. These results were confirmed by Oxnard study where diagnosis with both plasma- and tissue-based assays yielded equivalent outcomes after the institution of osimertinib treatment for T790M-mutant patients. Nevertheless, tumor biopsy is still needed to define the absence or presence of the mutation in patients with negative plasma results owing to a 30% false-negative rate. In addition to its diagnostic value, variations of urine circulating tumor DNA T790M mutation levels could be monitored to assess response and aid in guiding the treatment [20,21].

With the era of immunotherapy, cellular studies revealed an enhanced PD-L1 expression induced by EGFR activation, possibly inducing the apoptosis of T cells. Therefore, EGFR-TKIs are being tested as an option for enhancing antitumor immunity via PD-L1 downregulation, and by inhibiting tumor cell viability. In addition, anti-PD-1/PD-L1 antibodies could be of potential benefit when treating EGFR-TKI-sensitive patients and more importantly those with resistant-conferring mutations. Additional studies are needed to address the combination of EGFR-TKIs and anti-PD-1/PD-L1 antibodies [22]. The TATTON trial, a multiarm Phase Ib trial, addressed the combination of osimertinib with durvalumab (anti-PD-L1 monoclonal antibody) in patients with advanced NSCLC. Disease control rate was observed in more than 85% of patients, and consisted of partial response and stable disease. Most commonly reported side effects of any grade were vomiting, anemia and diarrhea. Interstitial lung disease, the most feared complication of checkpoint inhibitors, was reported in around 45% of patients, with grades 3 or 4 representing nearly the third. Therefore, combination strategies could provide a more durable response compared with TKI alone, but did not show any superiority in terms of overall response rate [23].

"Third-generation tyrosine kinase inhibitors are reserved for progression after previous tyrosine kinase inhibitor usage or for resistance-conferring mutations, mainly T790M. Moreover, it is expected in the near future that liquid biopsy will replace rebiopsy in the clinical practice."

To conclude, *EGFR* testing has become a standard in NSCLC treatment. Every patient presenting with a sensitizing *EGFR* mutation should be treated by a first- or second-generation TKI. Third-generation TKIs are reserved for progression after previous TKI usage or for resistance-conferring mutations, mainly T790M. Moreover, it is expected in the near future that liquid biopsy will replace rebiopsy in the clinical practice.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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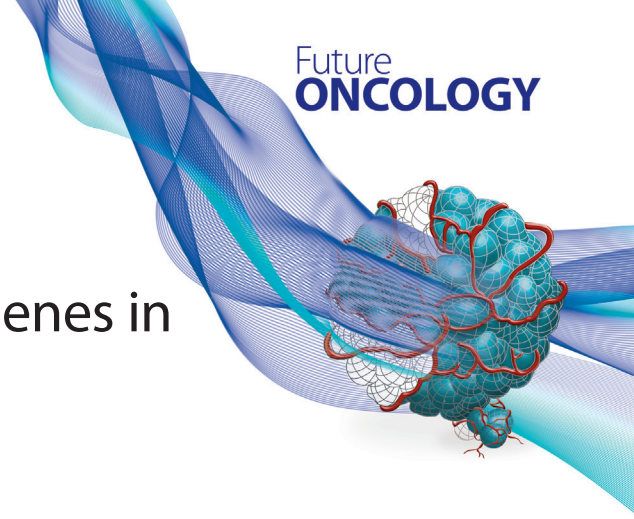

An integrated analysis of cancer genes in clear cell renal cell carcinoma

Jin Li[1], Liping Guo[2] & Zisheng Ai*[,3]

Aim: This study was performed to detect driver genes and implement integrated analyses on these drivers in clear cell renal cell carcinoma (ccRCC). **Methods:** Driver genes and pathways were predicted by OncodriveFM and Dendrix using 39,636 somatic mutations from The Cancer Genome Atlas, followed by DNA methylation, copy number variation, differential expression and survival analyses. **Results:** Overall, 342 driver genes and 106 pathways were determined by OncodriveFM, two driver genes by Dendrix. 28 driver genes were found hypomethylated, overexpressed and associated to a poor prognosis. By contrast, 17 driver genes showed decreased expression, hypermethylation and indicated a better outcome in ccRCC. **Conclusion:** The set of new cancer genes and pathways opens the avenue for developing potential therapeutic targets and prognostic biomarkers in ccRCC.

First draft submitted: 21 October 2016; Accepted for publication: 22 December 2016; Published online: 24 February 2017

KEYWORDS
• biomarkers/oncogenes/molecular oncology • genetics • molecular oncology

Renal cell carcinomas (RCCs) are one of the most common malignancies worldwide, with 271,000 new incidences and 116,000 deaths in 2008 [1]. Clear cell RCC (ccRCC) is the most aggressive and deadly subtype of RCCs and accounts for 70% of RCCs [2]. Even though remarkable advances have been achieved, the etiology of ccRCC is still not completely understood [3]. Driver genes which carry driver mutations play a pivotal role in the formation and progression of cancers and have become a focus of cancer genomics studies. An important strategy to detect driver genes focuses on genes that are significantly mutated in a cohort of cancer samples as compared with the background mutation rate [4,5]. Over the past 5 years, multiple tumor sequencing projects have been conducted to prioritize driver genes based on recurrent mutation status in a large number of ccRCC samples. VHL, PBRM1, BAP1, TCEB1 and SETD2 are the well-known frequently mutated driver genes in ccRCC [6,7]. However, many cancer genes that display middle or low mutation frequency might be neglected, such as genes that accumulate mutations with high functional impact (FI) and genes that are mutationally exclusive. To overcome these limitations, computational tools that could detect the middle- or low-frequency mutated driver genes have been developed, such as OncodriveFM [8] and Dendrix [9]. The former prefers to identify genes that show a bias toward accumulating variants with high FI predicted by three distinct tools SIFT [10], PolyPhen2 [11] and MutationAssessor [12]. The later aims to uncover sets of genes that show mutational exclusiveness in a cohort of cancer samples [9]. These tools provide a path toward mining potentially unknown cancer genes that drive cancer initiation and progression.

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In this study, we conducted a comprehensive study on the middle- or low-frequency mutated driver genes in ccRCC. Cancer driver genes were identified with OncodriveFM and Dendrix using somatic mutations detected by whole-exome sequencing of 499 normal/tumor pairs of ccRCC samples from The Cancer Genome Atlas (TCGA) database. In addition to previously reported driver genes, we revealed a list of new cancer-driving genes and pathways, including potentially druggable targets and prognostic biomarkers. We also found many driver genes that showed aberrant expression, DNA methylation levels, copy number variations and correlation with prognosis of ccRCC patients. Our study points out the importance of integrated genome, transcriptome and methylome analyses in characterizing cancer biology and identifying potential therapeutic targets in ccRCC.

Methods & materials

• Classification of cancer mutations

A total of 39,636 somatic mutations comprising 27,509 single-nucleotide variants (SNVs) and 12,127 small insertions or deletions (indels) were generated by whole-exome sequencing of 499 tumor/normal pairs and downloaded from TCGA ([13], download on 15 January 2016). Somatic mutations were classified into 12 categories based on their functional impact in the coding genome with Ensembl Variant Effect Predictor [14].

• Prediction of driver genes & pathways

Driver gene and pathway candidates were determined with OncodriveFM [8,15] and Dendrix [16] with default parameters following the criteria that genes and pathways have q values smaller than 0.05. To clarify the function enrichment of driver genes, GO enrichment analysis was carried out for all the driver gene candidates [17,18]. Then, ccRCC patients were stratified into HIF1a-deleted and HIF1a-expressed groups based on the occurrence of deletion (14q31.1) from TCGA database [19,20]. In total, 184 and 221 ccRCC patients were included in the HIF1a-deleted and HIF1a-expressed groups, respectively.

• Expression, DNA methylation & principal component analyses

Expression data were generated by RNA sequencing 65 pairs of ccRCC and normal tissues and obtained from TCGA. Differentially expressed genes were determined with *t*-test in R between ccRCC and normal tissues with the cutoff of p-value < 0.05. Next, principal component analysis (PCA) was applied to examine whether the differentially expressed genes and driver gene candidates could distinguish cancer from normal tissues. DNA methylation data of 283 ccRCC and 166 normal samples were accessed from TCGA, unavailable values were substituted with the mean β value.

• Sources of copy number variation & survival analyses

Copy number variations detected by single nucleotide polymorphism array were acquired from 528 ccRCC samples at broad institute [19,20]. TCGA RNAseq and clinical outcome data were retrieved to further elucidate whether driver genes could serve as prognosis biomarkers in ccRCC patients. For each driver gene candidate, ccRCC patients were divided into two groups, the 'high expression' and 'low expression' groups which refer to 25% ccRCC patients (130) that have the highest or lowest RNA expression levels of driver gene, respectively. Kaplan–Meier survival analyses were performed on patients in the high and low expression groups to assess the association between driver gene expression and clinical outcomes on the homepage of oncolnc [21,22].

Results

• The catalog of somatic mutations

A total of 39,636 somatic mutations comprising 27,509 SNVs and 12,127 indels generated by whole-exome sequencing of 499 pairs of ccRCC and normal samples were downloaded from TCGA. Indels, C>T/G>A, C>A/G>T and T>C/A>G, were the four most prevalent mutation types in ccRCC, with mutation rates 29.09, 21.69, 11.68 and 10.74% in the non-CpG sites, 1.5, 1.85, 0.83 and 0.5% in the CpG islands **(Figure 1A)**. There were 17,559 mis-sense mutations, 1247 nonsense mutations, 28 nonstop mutations, 6623 silent SNVs classified by Variant Effect Predictor. A total of 1852 deletions and 8445 insertions caused translational frameshifts, 530 deletions and 150 insertions were in frame mutations. A total of 1804, 968, 13 and 33 mutations occurred in splicing sites, RNA, intron and translation start sites, respectively. Over 70% (27,884/39,636) of total variants were nonsynonymous mutations **(Figure 1B)**. ccRCC had a lower nonsynonymous mutation density (0.62

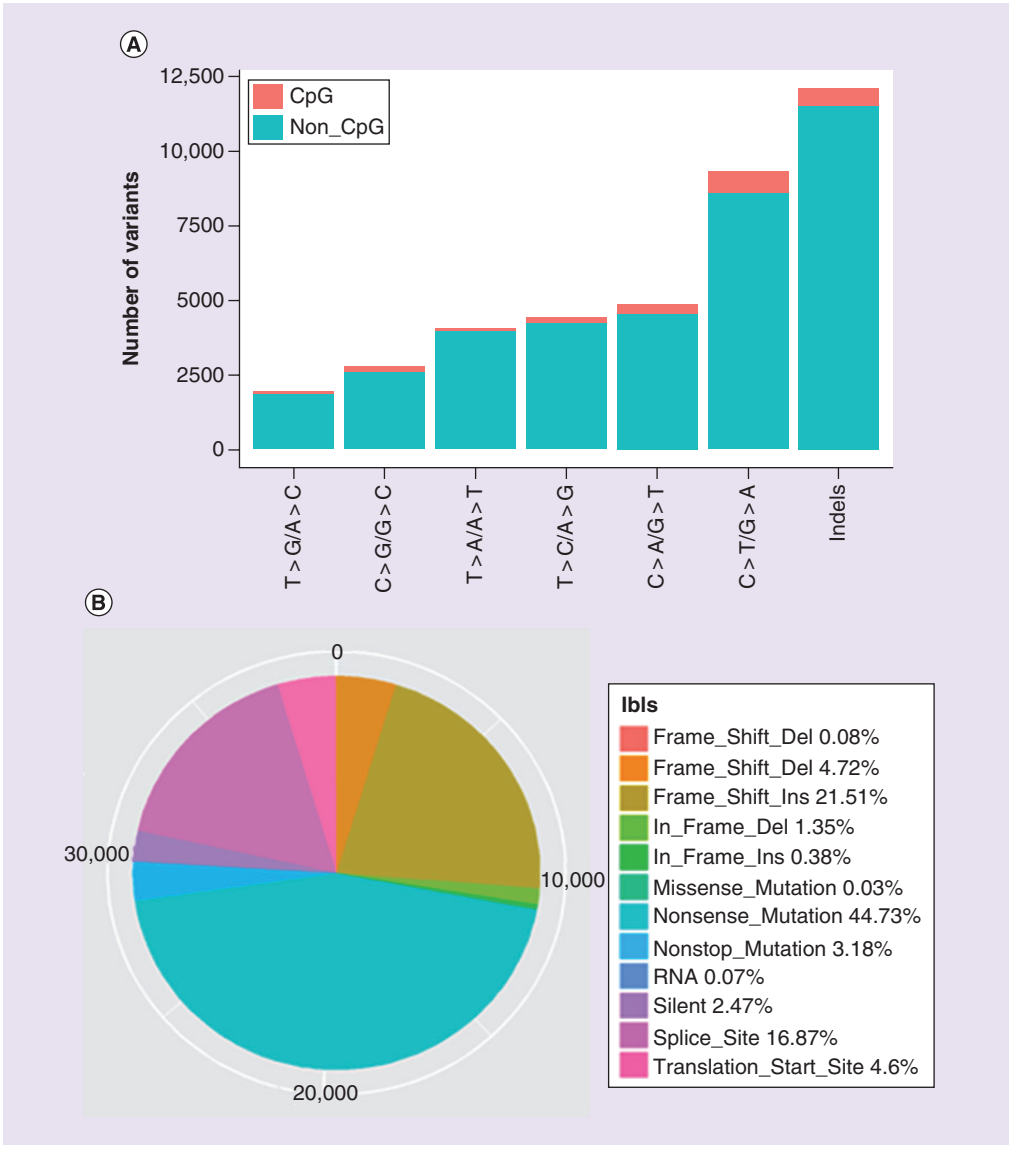

Figure 1. (A) Mutation signatures in clear cell renal cell carcinoma; (B) the number and proportion of 12 categories of somatic mutations in clear cell renal cell carcinoma.

nonsynonymous mutations per Mb per sample on average) in comparison with other cancer types, such as melanoma and lung cancer [5].

• Cancer driver genes & pathways in ccRCC

OncodriveFM was used to identify driver genes and pathways in ccRCC. In total, 342 driver genes were uncovered by OncodriveFM. Among them, VHL, PBRM1, SETD2, KDM5C, PTEN, BAP1, MTOR and TP53 were recurrently known mutated genes and showed mutation rates 46.29, 28.06,10.82, 5.41, 4.21, 8.22, 6.81 and 3.41% across all samples [7]. Whereas, the majority of driver genes are low-frequency mutated genes in ccRCC **(Figure 2 & Supplementary Table 1)**. Next, Dendrix was applied to identify sets of genes whose mutations are mutually exclusive in 499 ccRCC samples. Overall, Dendrix reported 13,763 genes mutated in at least one patient. We performed Dendrix analyses for sets of size ranging from two to four. When k = 2, the pair VHL and TTN was sampled 21.2% (212/1000) of the times. When k ≥3, no gene set had sample frequency greater than 5%. The pair (VHL and TTN) is the most prevalent gene set in the mutual exclusivity test. The results support that VHL plays a pivotal role in the tumorigenesis of ccRCC.

The enrichment of gene ontology (GO) terms was performed for 342 cancer gene candidates,

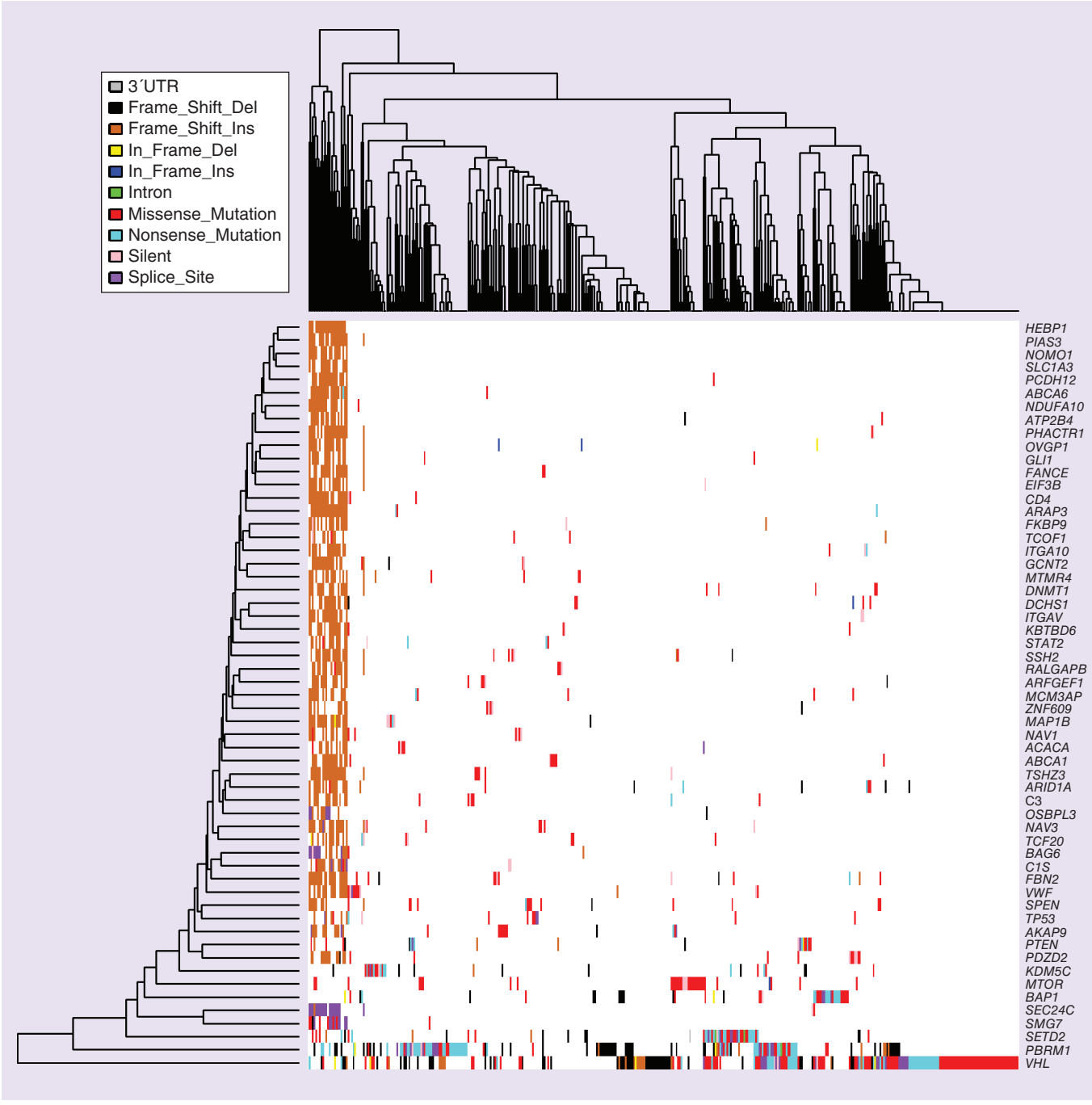

Figure 2. Mutation patterns of 57 cancer driver genes across 499 clear cell renal cell carcinoma samples.
UTR: Untranslated region.

59 GO terms were reported with significant statistical evidence. The GO terms showed a wide variety of functional processes ranging from regulation of protein metabolic process, regulation of cellular process, metabolic process, regulation of signaling, chromatin modification and chromatin organization. The results showed that these driver gene candidates contribute to tumorigenesis and progression of ccRCC mostly through involvement in metabolic processes, epigenetic modifications and regulation of cancer-associated signaling pathways in ccRCC. In addition, OncodriveFM also found 106 pathways with statistical FI bias in ccRCC, such as colorectal cancer, basal cell carcinoma, endometrial cancer, thyroid cancer, prostate cancer,

glioma, wnt signaling pathway, MAPK signaling pathway, P53 signaling pathway, PI3K–Akt signaling pathway, HIF-1 signaling pathway, mTOR signaling pathway, pancreatic cancer, RCC and melanoma **(Supplementary Table 2)**.

About 40% of ccRCC do not express HIF-1a gene, because of deletions involving 14q [7,23]. Therefore, the gene expression and the pathway activation may be influenced by the HIF status in ccRCC. We stratified ccRCC patients into HIF1a-deleted and HIF1a-expressed groups according to the occurrence of deletion (14q31.1). In total, 184 and 221 ccRCC patients were included in the HIF1a-deleted and HIF1a-expressed groups, respectively. Then we applied OncodriveFM and Dendrix to detect distinct driver genes and pathways in the two groups. As shown in **Supplementary Figure 1**, OncodriveFM unraveled 13 and 22 candidate driver genes, 33 and 49 driver pathways in the HIF1a-expressed and HIF1a-deleted groups, respectively **(Supplementary Tables 3–6 & Supplementary Figure 1)**. When k = 2, the pair DST and PTEN was sampled 34.5% (345/1000) of the times in the HIF1a-expressed group. No gene set had sample frequency greater than 5% in HIF1a-deleted group. Cancer driver genes and pathways were intersected among the HIF1a-expressed, HIF1a-deleted and whole ccRCC patient groups. We found seven overlapping driver genes, including ARID1A, BAP1, KDM5C, MTOR, PBRM1,SETD2 and VHL and 19 overlapping driver pathways, such as mTOR signaling pathway, pathways in cancer, RCC and gliomas among the three groups. Last, HIF1a-associated driver genes were also detected by OncodriveFM, including PRKCE in HIF1a-expressed group and POLR1A, PDS5B, NUDT21, WRN, LOXL3, ATM and EIF4A2 in HIF1a-deleted group **(Supplementary Tables 3–6 & Supplementary Figure 1)**.

• Expression profiling in ccRCC

To analyze the gene expression profile in ccRCC, we obtained RNA-seq data of 65 pairs of ccRCC and normal tissues. Overall, we found 12,430 differentially expressed genes between 65 pairs of ccRCC and normal samples. Among them, 202 are driver gene candidates. A total of 111 and 91 driver genes were upregulated and downregulated in ccRCC samples as compared with paired normal tissues, respectively **(Figure 3A)**. Next, PCA was applied to examine whether differentially expressed genes and driver gene candidates could discriminate cancer from normal tissues. As for the PCA results for differentially expressed genes, cancer tissues aggregated to the upper side, whereas normal tissues clustered to the lower side, however, the PCA results of driver gene candidates showed opposite distribution of ccRCC cancer and normal samples **(Figure 3B & C)**. All these results support that differentially expressed genes and driver genes could separate the tissue samples into two distinct groups.

• DNA methylation profiling in ccRCC

DNA methylation of cytosine within CpG dinucleotides maintains proper regulation of gene expression and stable gene silencing, therefore, DNA methylation alterations make a great contribution to tumorigenesis [24]. In order to elucidate the DNA methylation status of driver gene candidates, we downloaded DNA methylation data of 283 ccRCC and 166 normal tissues from TCGA and analyzed their association with driver genes in ccRCC. In total, 270 driver gene candidates showed aberrant DNA methylation levels between ccRCC and normal tissues **(Figure 3D)**. A total of 163 genes showed hypomethylation in ccRCC, such as POLR3E, MRPL24, RING1, RBM6, ACSL3, RQCD1, UST, HPGDS and SLC43A3, while, 107 genes had hypermethylation at their promoters, such as UHRF1BP1, EXTL2, PIGO, STAT2, BAZ2A, ALPK3, SP2, OSBP2, EPOR and IGFBP7 **(Figure 3D)**. These results suggest that driver genes might be implicated in ccRCC through modulating methylation status. In addition, 68 driver genes are overexpressed and hypomethylated, such as PHC2, PLD2, PLOD3, PLXNA2, POLE and POLR3E, 39 driver genes are lower expressed and hypermethylated, such as EIF4G2, EXTL2, FBN2, GAB1 GCNT2 and HSP90AA1, suggesting they might have oncogenic and tumor suppressor functions, respectively in ccRCC.

• Copy number variations in ccRCC

We also obtained copy number variations (CNVs) of 528 ccRCC samples detected by single nucleotide polymorphism array at broad institute.

Significant focal gains and deletions ($q < 0.25$) were found in 517 samples (517/528, 97.92%) at 29 loci (10 amplifications and 19 deletions). Among them, deletions at 3p22.2, 3p25.3 and amplification at 5q35.1 were the most frequent CNVs in ccRCC, with occurrence rate 88.01%

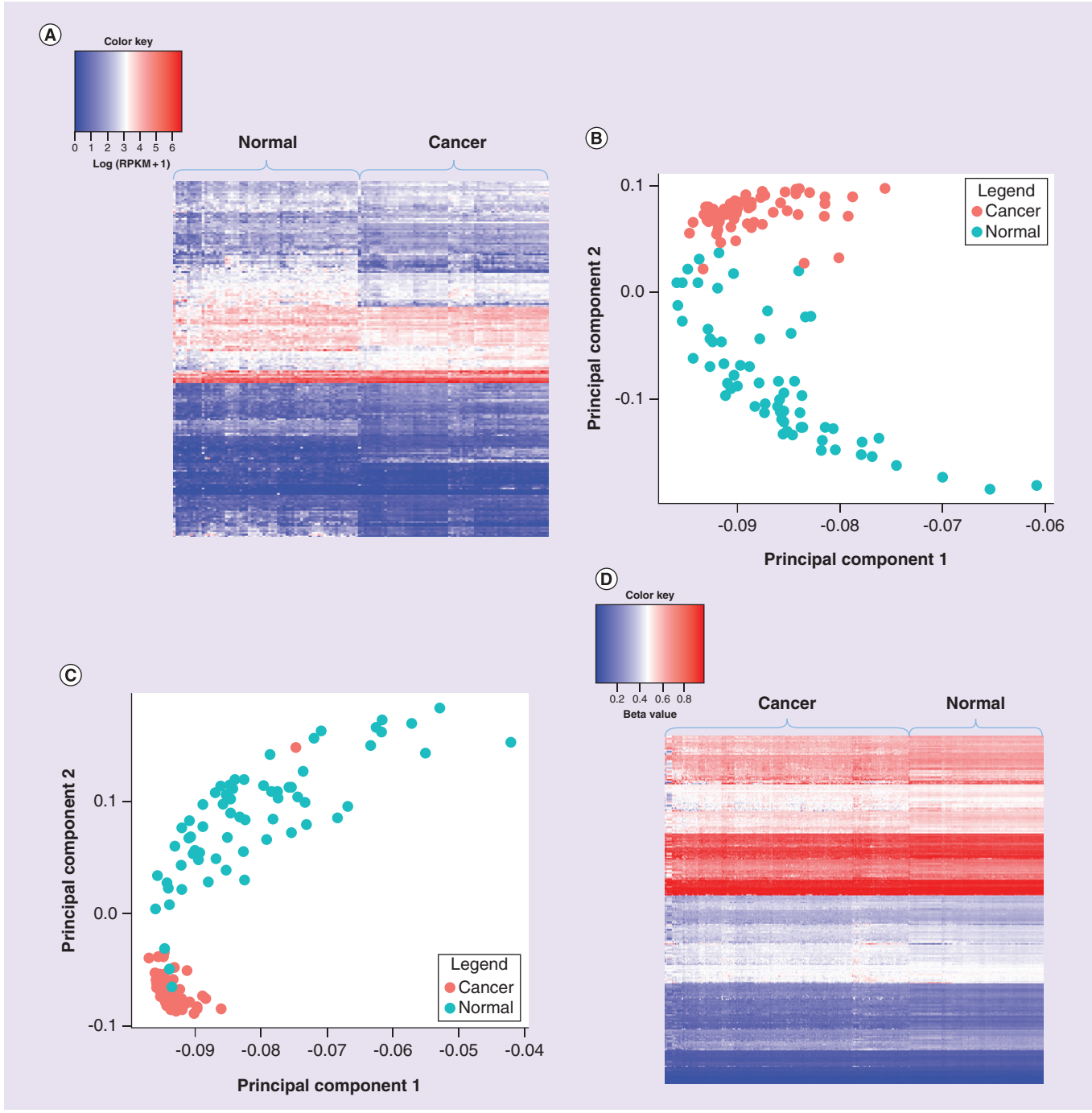

Figure 3. Expression and DNA methylation analyses of driver genes in clear cell renal cell carcinoma. (A) The expression difference of 202 driver candidates between clear cell renal cell carcinoma and normal samples, expression of each gene presented as RPKM was plus 1 and log scaled; **(B)** PCA of the 65 paired cancer and normal samples using 12,430 differentially expressed genes. Red and blue dots denote cancer and normal tissues, respectively. Each dot represents the expression values of the significant genes that were summarized at the first two principal component coordinates; **(C)** PCA of the 65 paired cancer and normal samples using 342 driver candidates. Red and blue dots denote cancer and normal tissues, respectively. Each dot represents the expression values of the 342 driver genes that were summarized at the first two principal component coordinates; **(D)** DNA methylation levels for 270 driver candidates, average β value was calculated for each gene and patient, undefined values were replaced with the mean.
PCA: Principal component analysis; RPKM: Reads per kilobase million.

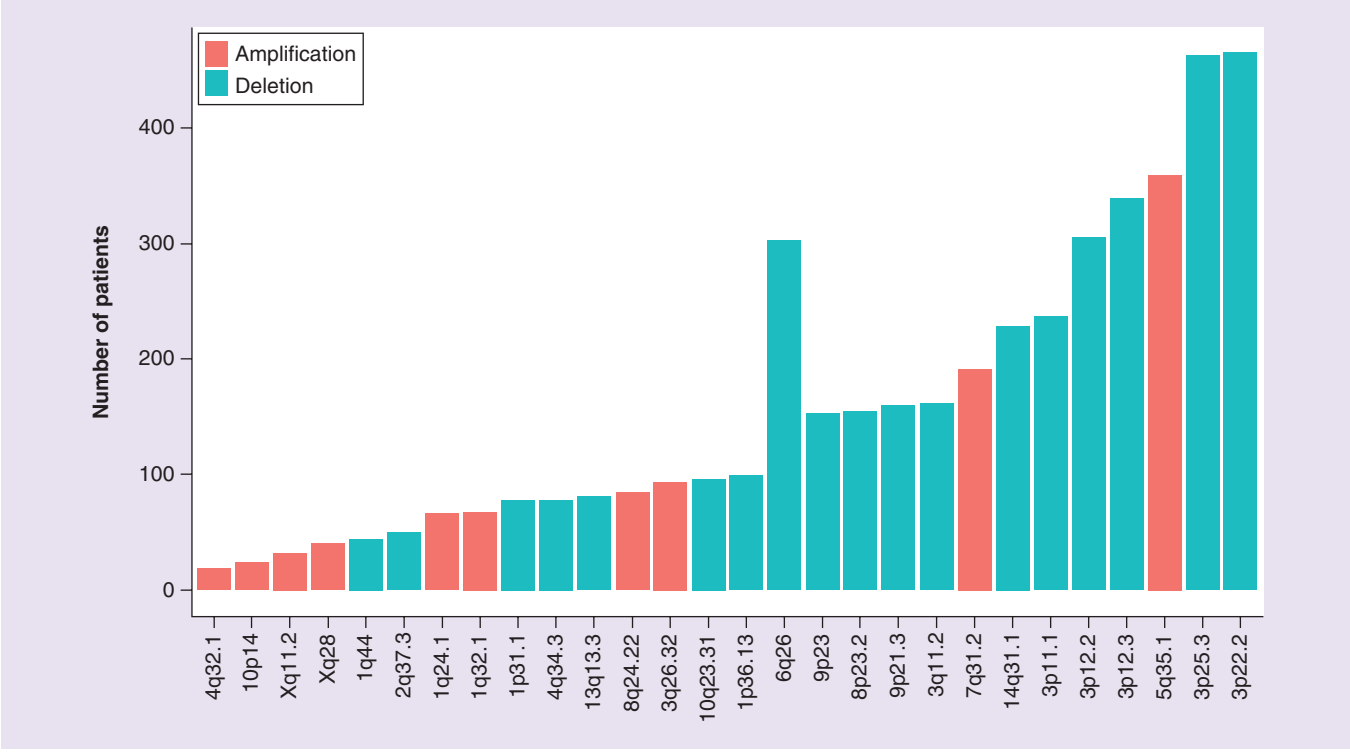

Figure 4. The number of copy number variations for each chromosome arm in 528 clear cell renal cell carcinoma samples.

(465/528), 87.69% (463/528) and 67.99% (359/528), respectively (Figure 4). A total of 55 cancer driver genes were involved in copy number variations, including known tumor suppressors and oncogenes, such as VHL (deletion, 3p12.3), PTEN (deletion, 10q23.31), MTOR (deletion 1p36.13). Many driver candidates were also found to be implicated in the CNVs, including MRPL24 (amplification, 1q24.1), FNDC3B (amplification, 3q26.32), PIK3C2B (amplification, 1q24.1, 1q32.1), SETDB1 (amplification, 1q24.1), PLXNA4 (amplification, 7q31.2), UST (deletion, 6q26), ARID1A (deletion, 1p36.13) and PDIA5 (deletion, 3q11.2).

• Survival analyses in ccRCC

We acquired TCGA RNAseq and clinical outcome data from TCGA to evaluate whether the expression of 342 driver genes is associated to survival and prognosis in ccRCC patients. Overall, Kaplan–Meier survival analyses showed that the expression of 186 driver genes was significantly associated to clinical outcomes of ccRCC patients. The high expression of 100 driver genes indicated a worse survival rate as compared with low expression groups, such as ZNF668, ZMYND19, ZFYVE27, ZCWPW1, HOXA3 and FXOM1 (Figure 5A). By contrast, patients with high expression of 86 driver genes showed better prognosis, such as ZNF347, ZNF175, ZEB1, MTOR, ZBTB5 and GAB1 (Figure 5B). A total of 28 driver genes were found hypomethylated, overexpressed and associated to a poor prognosis in ccRCC patients, such as DAGLB, DGKA, EIF3B, FAM104A and FOXM1. On the contrary, 17 driver genes showed decreased expression, hypermethylation and indicated a better outcome in ccRCC patients, such as GAB1, GCNT2, LMO7 and MTOR. These driver genes might be potential survival biomarkers for ccRCC patients in the future.

Discussion

In this study, we performed an integrated study on the 342 cancer driver genes and 106 pathways determined with OncodriveFM and Dendrix. Only a small fraction of these driver genes is recurrently mutated in ccRCC samples, such as VHL, PBRM1, SETD2, KDM5C, PTEN, BAP1, MTOR and TP53 [7]. Multiple known cancer genes of other cancer types were first identified as drivers in ccRCC, such as FGFR3 in bladder cancer [25,26], DNMT3A in acute myeloid leukemia [27], NF1 in glioma [28], TRRAP in Glioblastoma multiforme [29], NRF2

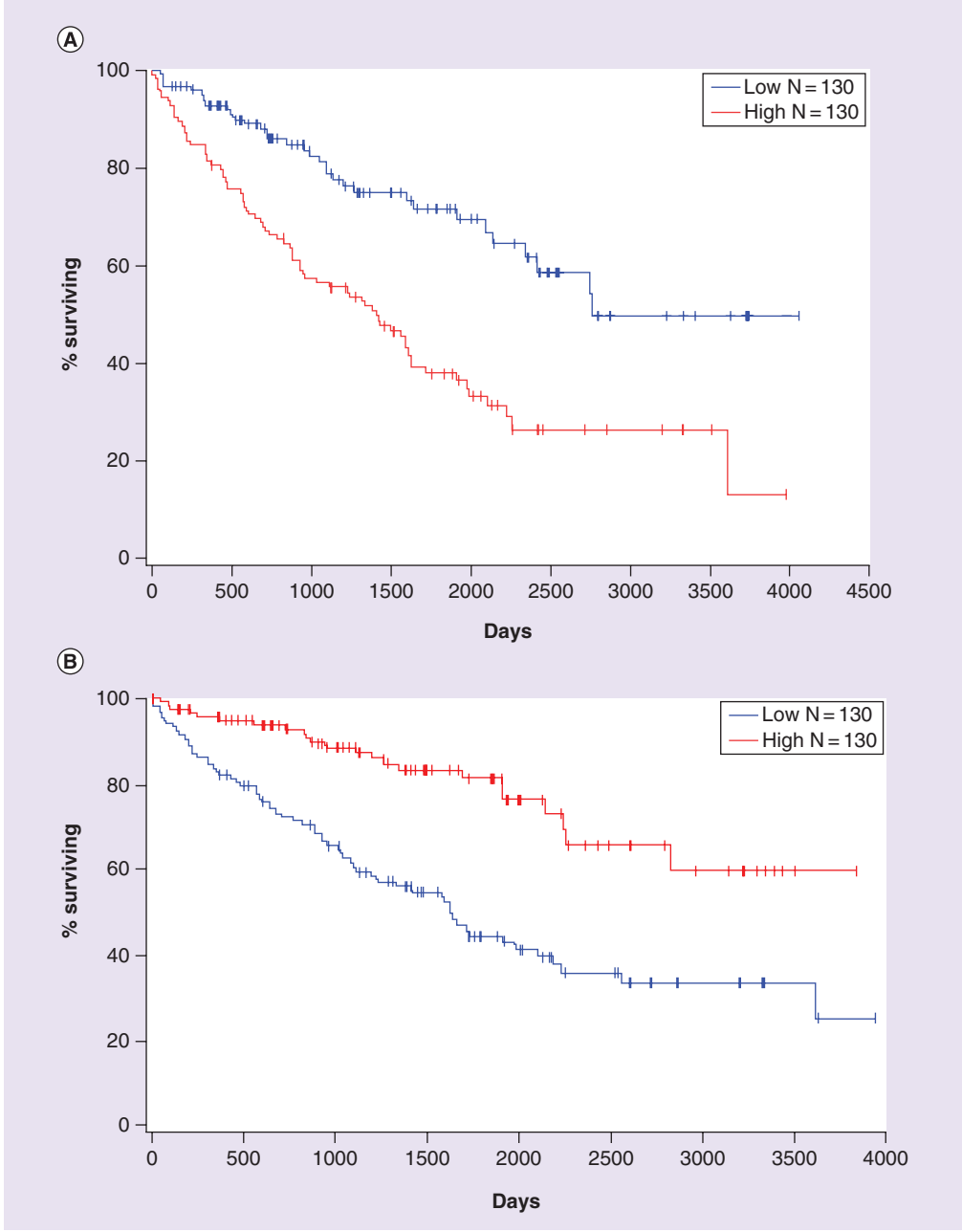

Figure 5. (A) Patients with high expression of FXOM1 (red) showed a poor survival rate as compared with those with low expression of FXOM1 (blue); (B) patients in the high expression group of GAB1 (red) showed a favorable prognosis than those in low expression group of GAB1 (blue).

in non-small-cell lung cancer (NSCLC) [30] and CSF3R in chronic neutrophilic leukemia and atypical (BCR-ABL1-negative) chronic myeloid leukemia [31]. We have also found many new driver candidates, for instance, LARP1 and RING1. LARP1 as a conserved RNA-binding protein interacts with poly-A-binding protein and modulates 5′-terminal oligopyrimidine tract mRNA translation. LARP1 expression was upregulated in cervical and NSCLCs, enhanced expression of LARP1 aggravated cell migration, invasion, growth and tumorigenicity *in vivo* through post-transcriptionally altering gene expression, such as mTOR in cancers [32]. RING1 is overexpressed in multiple cancer types, such as hepatocellular carcinoma

(HCC) [33], NSCLC [34], prostate cancer [35] and pancreatic cancer cells [36]. Silencing Ring1 expression caused growth inhibition and G1/S cell cycle arrest in HCC and NSCLC cells [33,34]. Knockdown of Ring1A and Ring1B reduced the binding of Snail to the target chromatin, eliminated H2AK119Ub1 modification, and impaired Snail-mediated transcriptional suppression and cell migration in pancreatic cancer cells [36]. In addition, RING1 interacts with multiple human PcG proteins, such as En-2, elevated levels of RING1 enhanced anchorage-independent growth of Rat1a fibroblast cells and formation of tumors in athymic mice by deregulating oncogenes expression, such as c-jun and c-fos [37]. In addition, multiple HIF1a-dependent driver genes were also detected by OncodriveFM, for instance, ATM. In the presence of hypoxia, ATM phosphorylates HIF-1a on serine^{696} and mediates downregulation of mTORC1 signaling that is critical to maintain normal cell growth and proliferation in solid tumor [38]. Therefore, deletion of HIF1a might disrupt the ATM-mediated regulation of mTORC1 signaling and initiate the formation of ccRCC.

The strength of OncodriveFM and Dendrix lies in the identification of cancer genes and pathways which display high FI bias toward accumulating high FI variants or mutational exclusivity, regardless of cancer mutation frequency. Implementing these tools allows for a better mining of cancer-driving genes and pathways in cancer genomics studies. In addition, we also uncovered 202 differentially expressed driver genes, of which 68 were overexpressed and hypomethylated, 39 lower expressed and hypermethylated as well as 55 cancer driver genes were involved in CNVs, suggesting these genes might contribute to initiation and progression of ccRCC in a variety of fashions.

Of the 342 driver gene candidates, we found 186 genes whose expression levels significantly related to ccRCC patient prognosis. A total of 28 driver genes were found hypomethylated, overexpressed and associated to a poor prognosis in ccRCC patients, such as DAGLB, DGKA, EIF3B, FAM104A and FOXM1. Take FXOM1 for example, FOXM1 is a key transcription factor regulating cell cycle progression, DNA damage response and a host of other hallmark cancer features. FXOM1 is overexpressed in multiple cancer types, such as leiomyosarcoma [39], B-lymphoblastic leukemia [40], gastric cancer [41], colon cancer [42], liver cancer [43] and melanoma [44]. The expression of FOXM1 is associated to a poor survival in many cancers. Knockdown of FOXM1 expression inhibits cellular growth in various cancer cells [39–41,45], suggesting it might function as an oncogene in cancers. In contrast, 17 driver genes showed decreased expression, hypermethylation and indicated a better outcome in ccRCC patients, such as GAB1, GCNT2, LMO7 and MTOR. Expression of GAB1 was highly and positively correlated with lymph node metastasis and TNM stage in intrahepatic cholangiocarcinoma tissues. Silencing GAB1 expression caused decreased cell proliferation, cell cycle arrest, increased apoptosis and decreased invasion in hilar cholangiocarcinoma cells [46]. GAB1 and Shp2 are involved in promoting invasion motility and tumor growth of Met overexpressing colorectal cancer cell line, DLD1 [47]. GAB1 overexpression promoted VEGF-induced endothelial cell migration. Knockdown of GAB1 Expression impairs VEGF-dependent signaling, migration and capillary formation [48]. In addition, GAB1 is a novel target gene of miR-409-3p which functions as a metastatic suppressor in colorectal cancer, and the metastatic suppression of miR-409-3p on colorectal cancer cells was caused by post-transcriptional inhibition of GAB1 [44]. Such driver genes as FXOM1 and GAB1 might provide potential prognostic biomarkers and targeted therapies for ccRCC patients in the future.

Conclusion

Taken together, we applied OncodriveFM and Dendrix to unravel a set of driver genes and made an integrative investigation on them, which strengthened our knowledge of tumorigenesis and progression of ccRCC. The driver genes and pathways identified herein, such as FXOM1 and GAB1 might be candidate biomarkers and drug targets for ccRCC prognosis and treatment.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: http://www.futuremedicine.com/doi/full/10.2217/fon-2016-0473

Authors' contributions

L Guo retrieved cancer mutations and RNA-seq data from TCGA, copy number variation from broad institute. J Li predicted cancer genes and pathways, conducted differentially expressed gene expression, PCA and survival analyses. Z Ai gave suggestions to the statistical analyses. J Li and Z

Ai drafted the manuscript. All authors read and approved the final manuscript.

No writing assistance was utilized in the production of this manuscript.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

Aim

- Clear cell renal cell carcinoma (ccRCC) accounts for 70% of RCCs and is one of the major causes of cancer-associated deaths worldwide.
- Though remarkable advances have been achieved, the etiology of ccRCC is still largely unknown.
- Detection of recurrently mutated driver genes has been the subject of cancer genomics studies, leading to the neglect of middle or low mutated drivers.

Methods

- Driver genes and pathways were predicted by OncodriveFM and Dendrix using 39,636 somatic mutations from The Cancer Genome Atlas, followed by DNA methylation, copy number variation, differential expression and survival analyses.

Results

- Overall, 342 driver genes and 106 pathways were determined by OncodriveFM, two driver genes by Dendrix.
- Twenty eight driver genes were found hypomethylated, overexpressed and associated to a poor prognosis.
- By contrast, 17 driver genes showed decreased expression, hypermethylation and indicated a better outcome in ccRCC.

Conclusion

- Our study pinpoints the importance of analyzing low or middle mutated driver genes in an integrative fashion.
- The driver genes and pathways identified herein, such as FXOM1 and GAB1 might be candidate biomarkers and drug targets for ccRCC prognosis and treatment.

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