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ORIGINAL ARTICLE

High selectivity of PI3Kβ inhibitors in SETD2-mutated renal clear cell carcinoma

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Summary

Purpose: *Clear cell renal cell carcinoma (ccRCC) is characterized with frequent mutations of SETD2 gene and our purpose was to explore targeted therapy for this entity.* ***Methods:*** *By bioinformatic investigation of two major databases, the Genomics of Drug Sensitivity in Cancer (GDSC) database and The Cancer Genome Atlas (TCGA) database, we identified the selective PI3Kβ inhibitors TGX221 and AZD6482 as selective inhibitors for ccRCC with SETD2 mutations, with AZD6482 additionally targeting PIK3CA and CDK6 mutations.*

Results: *Further investigation on AZD6482 profile revealed that mutations in RB1, KRAS, NRAS and APC contributed in drug resistance. Changes in both AZD6482-sensitive and -resistant gene sets showed limited impact on prognosis. Western blotting showed AZD6482 did not induce changes in a panel of major downstream effectors of AKT, but substantially increased PMS2 level. AZD6482 also selectively inhibited migration, invasiveness, and colony formation of ccRCC cells with SETD2 mutations. Integrative network analysis revealed complex interactions between these genes except SETD2.*

Conclusion: *AZD6482 is a novel inhibitor with high selectivity for ccRCC SETD2 mutations. Increased activity of PI3K/AKT/PMS2 could play a role in SETD2 mutated ccRCC.*

Key words: *clear cell renal cell carcinoma, PMS2, SETD2*

Introduction

ccRCC is the predominant subtype of RCC characterized by distinct underlying gene mutations [1]. Recent reports using novel sequencing techniques have identified inactivation of histone modifying genes, including PBRM1, BAP1, and SETD2, distinguishing a new subtype of ccRCC [2-4]. SETD2 is suggested to mediate DNA mismatch repair (MMR), homologous recombination repair (HRR), and DNA double-strand break (DSB) repair [5-8]. All this evidence indicates a role of SETD2 in DNA damage response (DDR).

Current targeted therapies for metastatic ccRCC are mainly tyrosine kinase inhibitors targeting angiogenesis rather than the cancer cell itself, conferring limited effect and intolerance due to major adverse events [9]. Application of rapalogs that targets the mechanistic target of rapamycin (mTOR) of the RCC cells appeared even less promising, prolonging roughly 4 months of overall survival (OS) [10]. Therefore, finding novel agents targeting signature mutations in ccRCC in the era of big data and next generation sequencing techniques could not only increase the treatment efficacy but reduce the off-target side effects as well. Taking advantage of recently released large databases of cancer genomics and drug sensitivity that encompass whole genome or exome sequencing, we are now able to inspect mutations or copy number variance (CNV) of most genes and screen for potential targeted agents.

Previous studies investigated two major cancer genetic databases, The TCGA and GDSC, and

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identified PI3Kβ inhibitor TGX221 as a potential therapeutic agent for SETD2-mutated ccRCC [11,12]. In order to further understand the mechanism of PI3K involvement in SETD2 gene function, we performed the current study investigating the PI3Kβ inhibitor AZD6482 in ccRCC with SETD2 mutation.

Methods

Data mining and analysis of TCGA and GDSC database

Current targeted therapies of metastatic ccRCC conferred limited improvement to survival and could easily induce drug resistance. Also, the first-line targeted agents for ccRCC targeted the neovascularization but not the tumor cells themselves. With the scope of minimizing off-target effects and find potent tumor inhibitors, we studied the GDSC database to find potential selective compounds. We searched drugs with significant selectivity to the recently found frequently mutated genes in ccRCC including SETD2, BAP1, and PBRM1, whilst solely SETD2 mutation was found in the database.

Reproduction of TCGA and GDSC databases was reported previously [11]. Full permission to reproduce the data was acquired from submitter of each database [13-15]. The TCGA renal clear cell carcinoma (KIRC) database was analysed on the cBioPortal platform (http://www.cbioportal.org/). We captured data from the Provisional subset, which contained 537 samples in which 413 tumors were completely analysed for somatic mutations, putative copy-number alterations, mRNA expression by RNA-seq, and protein/phosphoprotein level detected with reverse phase protein array (RPPA) [16,17]. The GDSC project is a collaboration of multiple institutes that investigated cancer genomics for sensitivity to a variety of compounds and data released are fully accessible and reproducible [15]. As per our previous report [11], we analysed the online GDSC data processing platform [18], where the volcano plots, scatter plots, and the Mann-Whitney-Wilcoxon (MWW) tests were generated.

Cell culture and proliferation

The A498 and 786O renal cancer cells were acquired from the American Type Culture Collection (ATCC) and were cultured in RPMI-1640 medium with 20% foetal bovine serum (Gibco, Carlsbad, CA, USA). AZD6482 was acquired from Santa Cruz Biotechnology,Inc. (Santa Cruz, CA, USA). Proliferation was studied using crystal violet assay as previously reported [11]. Briefly, cells were seeded in 96-well plates and were treated with single dose of drug or control agent. Cells were then cultured for 72 hrs and subjected to formalin fixation. After several washes, cells were stained using crystal violet, which were later dissolved using methanol. Proliferation was profiled by absorption at 540 nm read on plate reader.

Western blotting

Blotting was performed as per previous reports [19,20]. Total protein of lysates was extracted and loaded onto 10% sodium dodecyl sulphate polyacrylamide gel for electrophoresis. The separated proteins were transferred to nitrocellulose membrane. The membranes were blocked for 1 h with 5% non-fat milk. The primary antibodies SETD2 (Abnova, Taipei, Taiwan), p/tAKT (S473, Cell Signaling, Danvers, MA, USA), PMS2 (Abcam, Cambridge, MA, USA), MDM2 (Abcam), pBAD (S99, Abcam), pNFκB (Santa Cruz), pmTOR (Cell Signaling), pGSK3β (Cell Signaling), pFKHR (Sigma Aldrich, Shanghai, China) were then added and membranes were kept at 4°C overnight. Horseradish-coupled secondary antibodies (Cell Signaling) were used to detect the bands.

Migration and invasion assay

Migration and invasion assay were performed according to previous reports. Briefly, 786O and A498 cells were seeded in the top chamber of the Transwell inserts, which were coated with Matrigel for invasion assay and uncoated for migration assay. Complete medium was added to the lower chamber and cells were then incubated. Inserts were harvested and cells penetrated the membrane were stained with crystal violet and were counted microscopically. The average number of cells in three high power fields was calculated. All assays were done in triplicate.

Colony formation assay

Soft agar assay was used to study the ability of colony formation of cells. Briefly, 1,000 786O or A498 cells were resuspended in complete medium mixed with 0.4 % Noble agar. The mixture was placed on top of the similar mixture consisting of complete medium and 0.6% of agar on 60-mm plates. Complete medium containing treatment and control drug was added at the upmost layer and was changed every 3 days. After 2 weeks of culture, plates were stained with crystal violet and colonies were counted microscopically.

Statistics

In silico statistical analysis of the TCGA data was automatically performed by the cBioPortal platform. For mRNA data and protein and phosphoprotein data, z scores were precomputed from the expression values. The z-scores for mRNA expression were determined for each sample by comparing a gene's mRNA expression to the distribution in a reference population that represented typical expression for the gene. If expression data were available for normal adjacent tissues, these data were used as the reference population; otherwise, expression values of all tumors that were diploid for the gene were used. For each available protein or phosphoprotein, cBioPortal performed a two-sided, two-sample Student's t-test to identify differences in

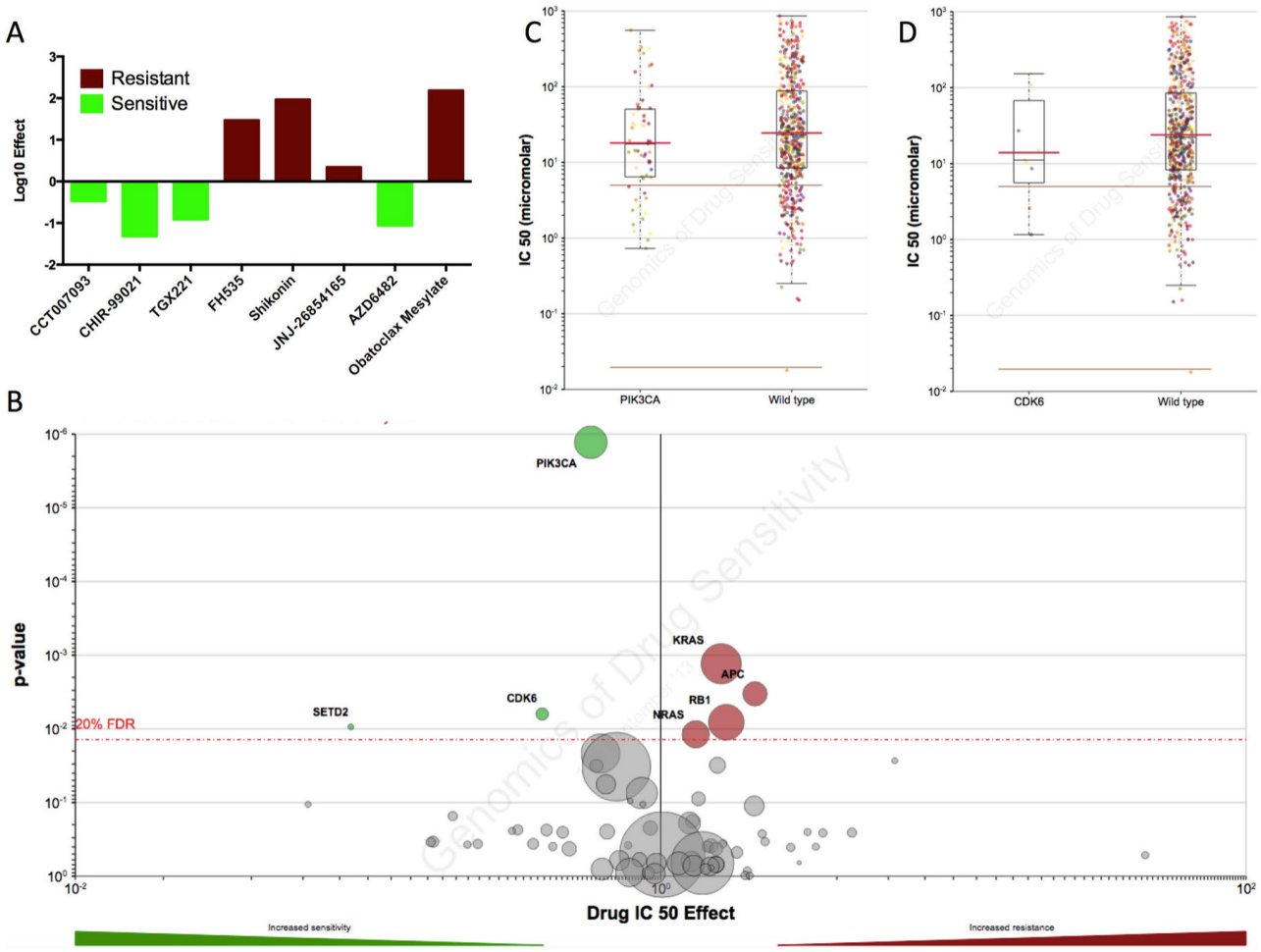

Figure 1. RCC cells with mutant SETD2 were sensitive to AZD6482, reproduced from the Genomics of Drug Sensitivity in Cancer (GDSC) database. **(A)**: Compounds sensitive and resistant to SETD2 mutation; **(B)**: Volcano plotting showing gene mutations marked in green as sensitive to AZD6482; Scattered plotting showing proliferation of cancer cells with **(C)** PIK3CA or **(D)** CDK6 mutation.

protein abundance between tumor samples that had at least one event (alteration) in one of the query genes. The results were displayed as a list of proteins or phosphoproteins, ranked by their difference in abundance between altered and unaltered samples. In the co-expression analysis, the Pearson's correlations were computed first. For genes with a correlation greater than 0.3 or less than -0.3, the Spearman's correlations were also computed. In our study, only gene pairs with values > 0.3 or <0.3 in both measures were accepted. All *in vitro* assays were run in triplicate in three independent experiments. A p value of <0.05 was accepted as statistically significant.

Results

Cancer cells with mutated SETD2 are sensitive to AZD6482

There were 4 hits for SETD2 mutation, among which both TGX221 and AZD6482 were PI3Kβ inhibitors (Figure 1A). We then studied the tissue specificity of AZD6482 and found that only renal cell carcinoma harbors SETD2 mutation in the GDSC database. In addition, we found that AZD6482 could also selectively inhibit cancer cells with mutations in PIK3CA and CDK6 (Figure 1B-D). These two genes also played critical roles in cancer development. Together, we found that AZD6482 selectively inhibits cancer cells with SETD2, PIK3CB and CDK6 mutations. These findings encouraged us to further study the mutation frequencies of these genes in ccRCC patients.

Genetic alteration in SETD2, PIK3CA and APC are common in ccRCC

It was well established that VHL mutations played pivotal roles both in hereditary and sporad-

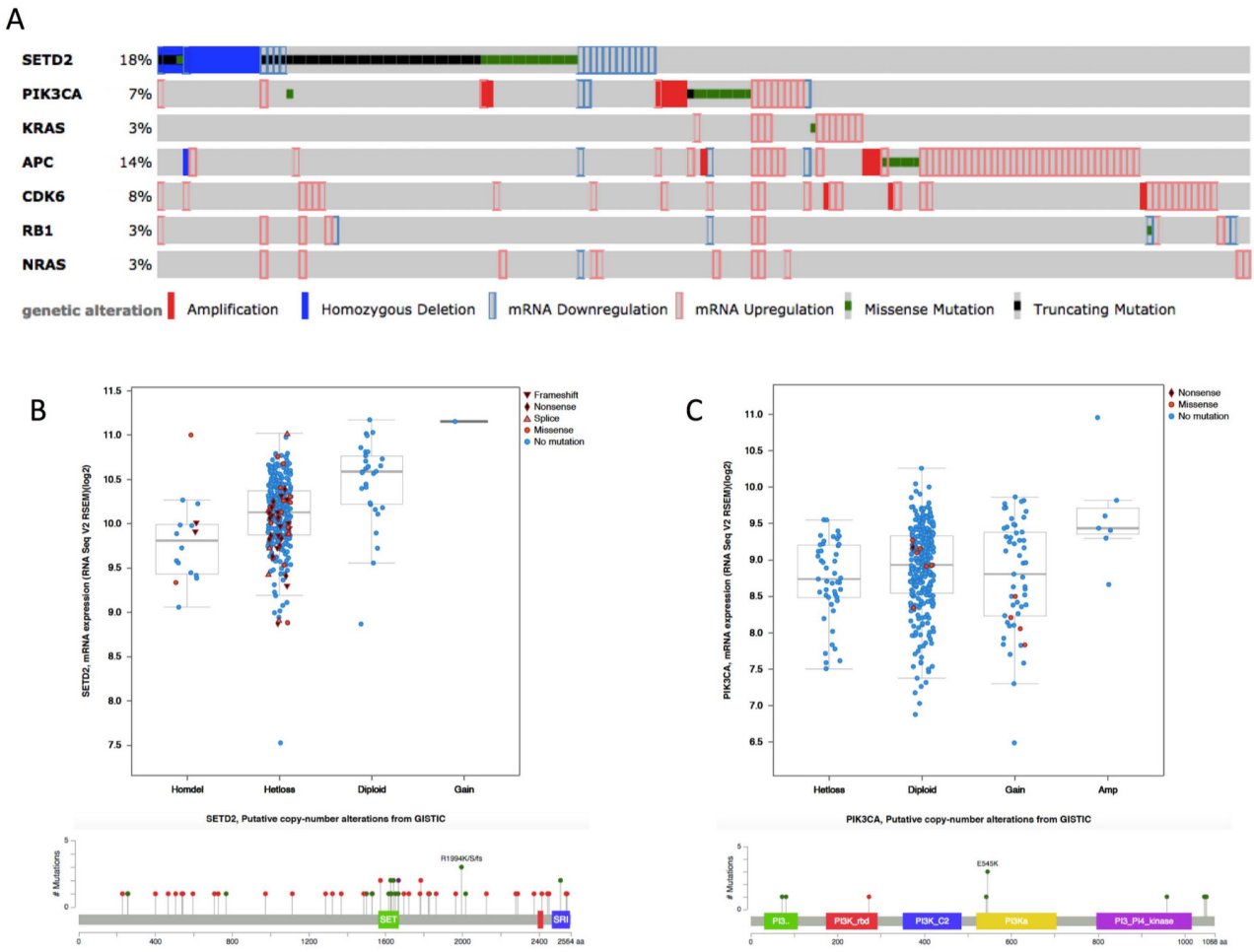

Figure 2. Somatic mutation in SETD2 is common in renal cell carcinoma. Reproduction of The Cancer Genome Atlas (TCGA) database showing **(A)**: frequencies and types of mutations in SETD2 and other AZD6482 sensitivity associated genes in ccRCC patients. Correlation between mRNA expression measured by RNA-seq and copy number variance of **(B)**: SETD2, and **(C)**: PIK3CA with lower boxes showing mutation loci within each gene.

ic ccRCC. Recent studies discovered high frequency of mutant SETD2, PBRM1, and BAP1, which were involved in chromatin modulation ccRCC as well [3]. Our reproduction of TCGA data confirmed frequent genetic alteration in SETD2 status by mutation, CNV or downregulation (~18%) in ccRCC (Figure 2A). We also found that truncating mutations were predominant in SETD2 (Figure 2A). We then studied genetic alterations in all genes associated with AZD6482 sensitivity as shown in GDSC (Figure 1B). Mutation and arm level changes were more commonly seen in PIK3CA, CDK6 and APC. Expressions of these genes were generally upregulated in ccRCC (Figure 2A). However, mutation and CNV in KRAS, NRAS, and RB1 were uncommon. For SETD2 and PIK3CA, deletion and amplification were associated with decreased and increased mRNA expression, respectively (Figure 2B,C). We showed that SETD2 mutation and deletion were common in ccRCC, and even the unchanged cases could show downregulation of the gene. Though not as common, changes in PIK3CA and APC and CDK6 were mainly amplification and upregulation.

AZD6482 –sensitive and –resistant gene set in ccRCC

As mentioned before, off-target effects generated unfavorable adverse events that could often be so intense that targeted treatment in mRCC patients was forced to cease [21]. We therefore studied the clinical contributions of targets other than SETD2 in ccRCC. Interestingly, neither the AZD6482–sensitive (SETD2, PIK3CA, and CDK6) nor –resistant (RB1, NRAS, KRAS, and APC) gene

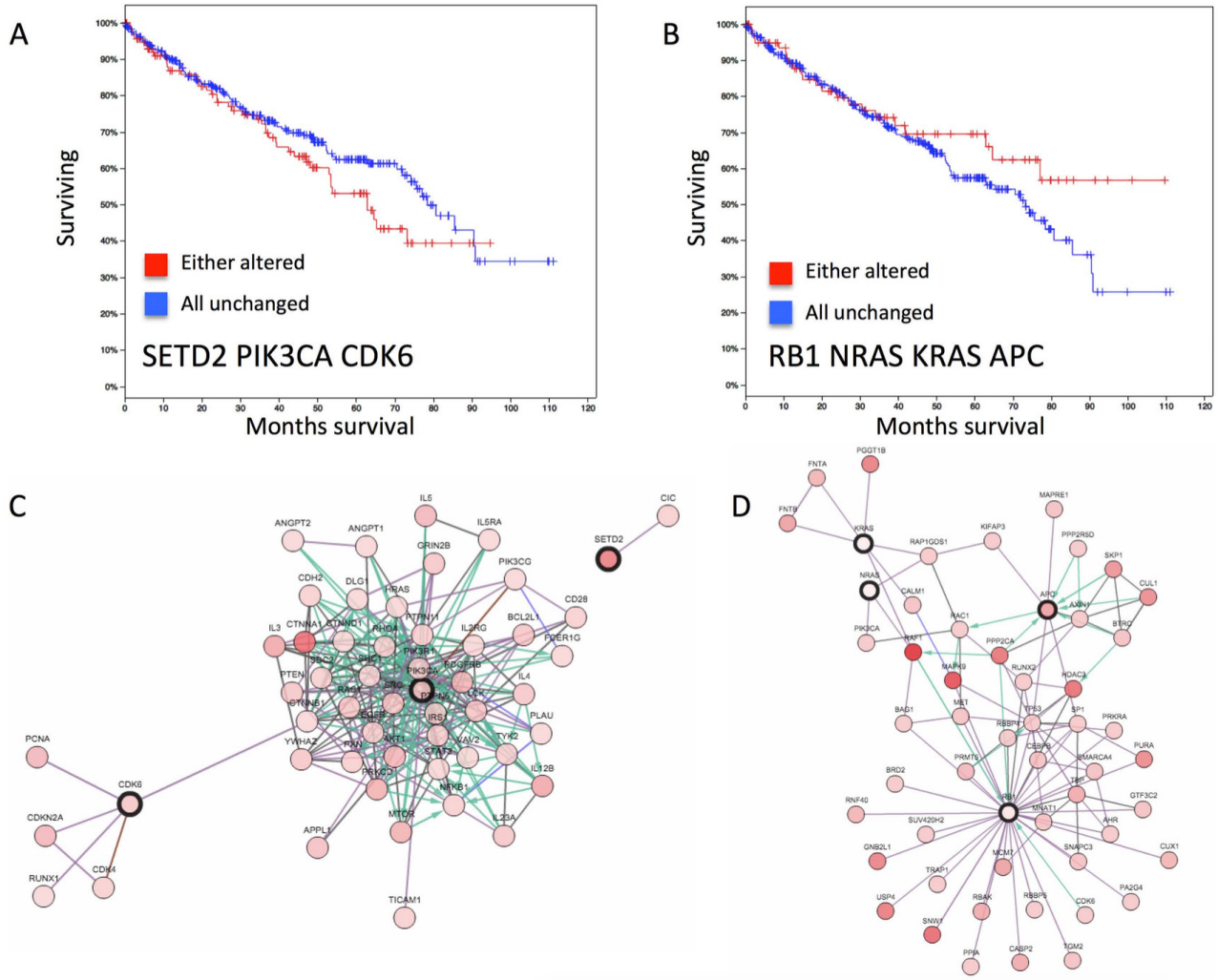

Figure 3. AZD6482–sensitive and –resistant gene set in ccRCC. Reproduction of The Cancer Genome Atlas (TCGA) database showing **(A)** neither the AZD6482–sensitive (SETD2, PIK3CA, and CDK6) nor **(B)**–resistant (RB1, NRAS, KRAS, and APC) gene set changes were associated with prognosis. Network analysis showing interactions between AZD6482 –sensitive **(C)** (SETD2, PIK3CA, and CDK6) and resistant **(D)** (RB1, NRAS, KRAS, and APC) gene set.

set changes were associated with prognosis (Figure 3A,B). We then used network analysis to investigate the potential link between the genes within the gene set. Interestingly, no solid association between SETD2, PIK3CA, and CKD6 was observed in ccRCC (Figure 3C). Nonetheless, although less commonly altered in ccRCC, there were more lucid associations between the genes (Figure 3D). We noticed that alteration in AZD6482 sensitivity-related gene sets in ccRCC did not impact prognosis. The fact that SETD2 mutated cells were sensitive to PI3Kβ inhibitor and lacked direct gene-gene interaction between SETD2 and PIK3CA indicated a more complex signalling.

AZD6482 selectively inhibits RCC with mutated SETD2

As per our results computed from 2 solid databases, we further studied the effect of AZD6482 on kidney cells with different genetic background. We first confirmed that A498 RCC cell line harbored mutation in SETD2 and 786O had wild-type SETD2 (Figure 4A). We then found that AZD6482 decreased AKT phosphorylation in A498 cells but not in 786O cells (Figure 4A). We therefore studied a panel of well-established downstream effectors of AKT. Interestingly, only PMS2 showed substantial changes in SETD2 mutated A498 cells treated by AZD6482 (Figure 4A). AZD6482 demonstrated strong selectivity for A498 cells in comparison to 786O cells with regard to proliferation (Figure 4B). The inhibitory effect of AZD6482 in both migration and invasiveness were also significant in A498 cells, whereas in 786O cells the inhibition

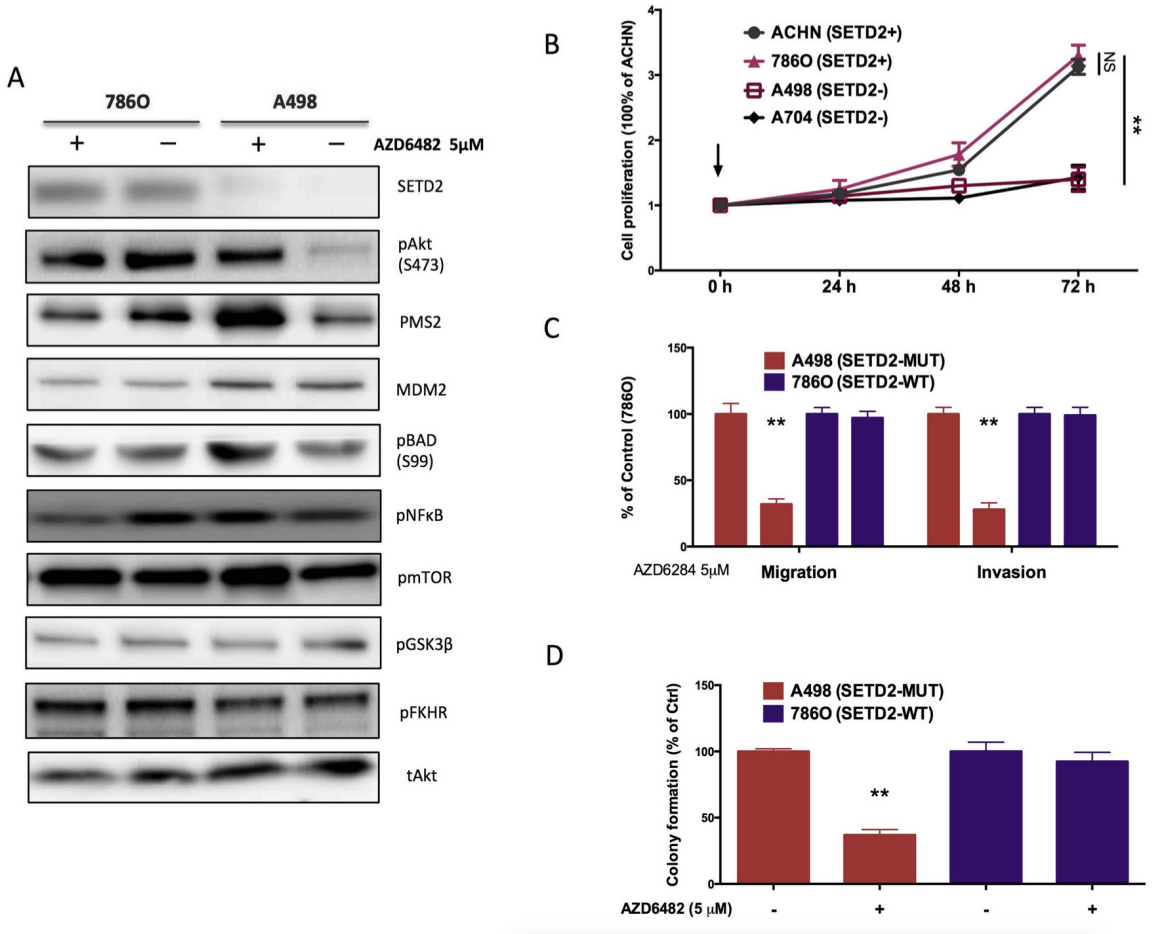

Figure 4. TGX221 selectively inhibits ccRCC with mutated SETD2. **(A)**: Application of AZD6482 in SETD2-wild type 786O cells and in SETD2-mutated A498 RCC cells showing selective inhibition of phosphorylation of AKT and PMS2 in A498 cells; Compared with 786O cells, TGX221 selectively inhibits the **(B)** proliferation; **(C)** invasiveness and migration, and **(D)** colony formation in A498 cells (**p < 0.01).

was insignificant (Figure 4C). Similarly, AZD6482 did not inhibit the colony formation of 786O cells but significantly decreased colony forming ability of A498 cells (Figure 4D). Together, we used *in vitro* assays to confirm that AZD6482 selectively inhibited RCC cells with SETD2 mutation.

Discussion

In the current study, we have used 2 major databases to identify a compound, AZD6482, that is not previously studied in ccRCC and has strong selectivity for tumors having SETD2, PIK3CA, and CDK6 mutations. Distinctive from anti-angiogenic agents that target the neovasculature resultant from uncurbed HIF expression in VHL mutated cases, a compound that targets the somatic mutation of RCC tumor cell itself appears intriguing. It is confounding that the selectivity for SETD2 mutation could be conferred by PI3Kβ inhibitors in RCC. Amongst the 4 most common gene mutations located on 3p, SETD2 together with PBRM1 and BAP1 has merely been identified recently. SETD2 functions in the epigenetic regulation of a series of gene expression in, but not limited to RCC [22,25]. In another study that also reproduced TCGA KIRC data, SETD2 was associated with worse cancer specific survival (CSS) [23]. Lack of insight of the biological effect of SETD2 solely entitles it as a novel class disease marker and less as a treatment target in ccRCC. To date, there have not been reports on the targeted therapy for SETD2 in any cancer types, let alone RCC. Therefore, the selectivity of PI3Kβ inhibitors for SETD2 implies an inherent connection between SETD2 and PI3K signalling.

Besides the selectivity TGX221 also targets PIK3CA and CDK6 mutations that, albeit not com-

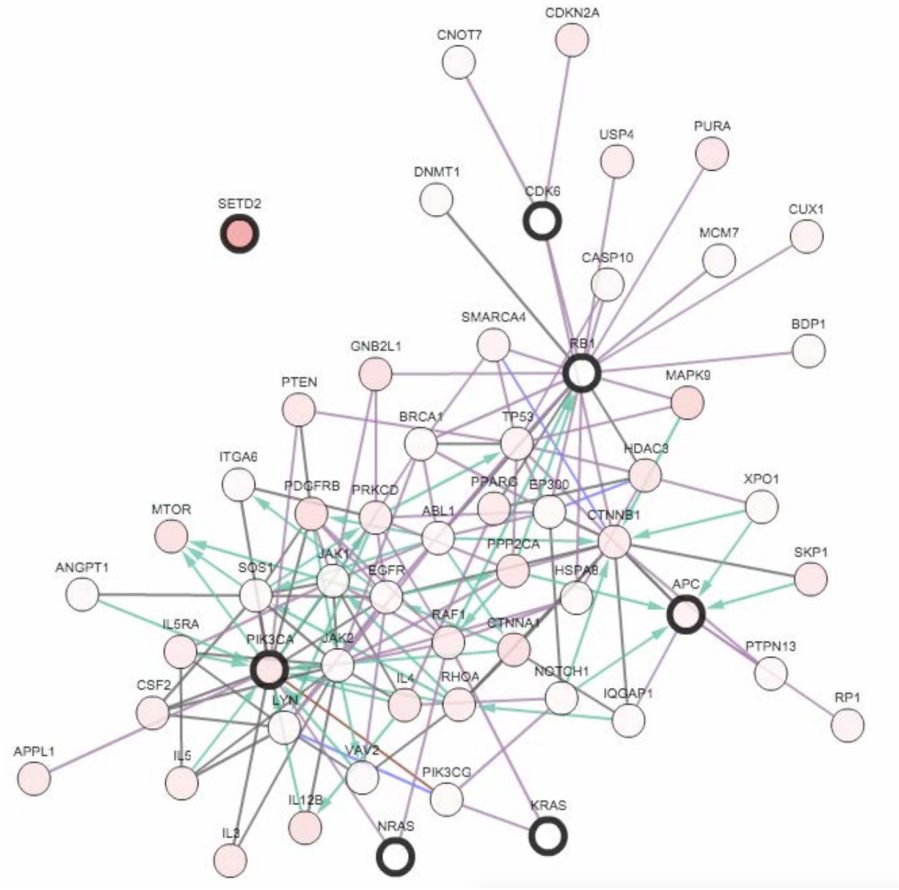

Figure 5. Network analysis from The Cancer Genome Atlas (TCGA) database showing that a complicated network existed connecting PIK3CA, CDK6, RB1, NRAS, KRAS, and APC, whilst leaving SETD2 unrelated.

mon in ccRCC, play critical roles in a variety of cancers [24,25]. Selective PI3Kβ inhibitors, like TGX221 and KIN193, are known to be sensitive in PTEN mutant cells and are thought to minimize the side effects caused by pan-PI3K inhibitors and p110α-selective inhibitors [26,27]. It is thus intriguing that PTEN was merely mutant in 4% of ccRCC patients [13], whereas TGX221 has not been reported to be selective to other mutations. Also, mutation in PIK3CB was only detected in 1 patient in TCGA KIRC, in contrast to the commonly altered PI3K pathway in which PIK3CA had high-level amplification [2]. In our study, PIK3CA was shown to be altered in ~7% of ccRCC patients. Although the frequency for mutation/CNV was even lower, we surmise that SETD2 is implicated in the PI3K signalling and have subsequently found that PMS2 was associated with PI3K activity. As one of the critical factors in the MMR machinery, lowered PMS2 could potentially lead to impaired integrity and functional loss of MMR. SETD2 has been shown to be indispensable for the localization or the critical MMR component MSH6. Lack of SETD2 and the resultant decreased trimethylation of H3K36 may cause impaired MMR machinery [6]. Previous reports showed that in ccRCC with silenced SETD2, cases with SETD2 deletion were in close association with loss of MLH1, another MMR component, which interacts with PMS2 [11,12]. Feng et al. also showed that loss of MLH1 could confer resistance to TGX221. In reminiscence of the recent studies [7,11,12] it is possible that SETD2 is associated with MMR and genomic stability via multiple routes. In order to validate our speculation, we performed the computed network analysis from KIRC, which shows in the core of the network RB1 and PIK3CA building up interactions between CDK6, APC, NRAS, KRAS but not SETD2 (Figure 5). These deductive or computed interactions, though intuit us how these genes could interact in ccRCC to maintain tumor characteristics, still warrant insightful

studies to elucidate both the pharmacological mechanisms of AZD6482 and other PI3Kβ inhibitors and the interplays between common genetic and genomic variances in kidney cancer.

Conclusion

ccRCCs have been characterized with frequent mutation in SETD2. We have found via GDSE database that AZD6482 is a selective inhibitor for cancer cells with SETD2 mutation. Further study showed that AZD6482 significantly inhibits ccRCC with SETD2 mutation. Increased PI3K/AKT/PMS2 activity could play a role in SETD2 mutated ccRCC, indicating the involvement of MMR impairment in the setting of SETD2 mutation.

References

1. Linehan WM. Genetic basis of kidney cancer: role of genomics for the development of disease-based therapeutics. Genome Res. 2012;22:2089-2100.
2. Guo G, Gui Y, Gao S et al. Frequent mutations of genes encoding ubiquitin-mediated proteolysis pathway components in clear cell renal cell carcinoma. Nat Genetics 2012;44:17-19.
3. Dalgliesh GL, Furge K, Greenman C et al. Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. Nature 2010;463:360-363.
4. Varela I, Tarpey P, Raine K et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 2011;469:539-542.
5. Carvalho S, Vitor AC, Sridhara SC et al. SETD2 is required for DNA double-strand break repair and activation of the p53-mediated checkpoint. eLife. 2014;3:e02482.
6. Li F, Mao G, Tong D et al. The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSalpha. Cell 2013;153:590-600.
7. Schmidt CK, Jackson SP. On your mark, get SET(D2), go! H3K36me3 primes DNA mismatch repair. Cell 2013;153:513-515.
8. Pfister SX, Ahrabi S, Zalmas LP et al. SETD2-dependent histone H3K36 trimethylation is required for homologous recombination repair and genome stability. Cell Rep 2014;7:2006-2018.
9. Hwang C, Heath EI. The Judgment of Paris: treatment dilemmas in advanced renal cell carcinoma. J Clin Oncol 2014;32:729-734.
10. Stein A, Bellmunt J, Escudier B et al. Survival prediction in everolimus-treated patients with metastatic renal cell carcinoma incorporating tumor burden response in the RECORD-1 trial. Eur Urol 23;64:994-1002.
11. Feng C, Ding G, Jiang H, Ding Q, Wen H. Loss of MLH1 confers resistance to PI3Kβ inhibitors in renal clear cell carcinoma with SETD2 mutation. Tumor Biol 2015;36:3457-3464.
12. Feng C, Sun Y, Ding G et al. PI3Kbeta inhibitor TGX221 selectively inhibits renal cell carcinoma cells with both VHL and SETD2 mutations and links multiple pathways. Sci Rep 2015;5:9465.
13. Cancer Genome Atlas Research. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 2013;499:43-49.
14. http://cancergenome.nih.gov/publications/publicationguidelines
15. Garnett MJ, Edelman EJ, Heidorn SJ et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. Nature 2012;483:570-575.
16. Gao J, Aksoy BA, Dogrusoz U et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Sign 2013;6:l1.
17. Cerami E, Gao J, Dogrusoz U et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401-404.
18. Yang W, Soares J, Greninger P et al. Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. Nucleic Acids Res 2013;41(Database issue):D955-61.
19. Ding G, Feng C, Jiang H et al. Combination of Rapamycin, CI-1040, and 17-AAG Inhibits Metastatic Capacity of Prostate Cancer via Slug Inhibition. PloS One 2013;8(10):e77400.
20. Feng C, Wu Z, Guo T et al. BLCA-4 expression is related to MMP-9, VEGF, IL-1α and IL-8 in bladder cancer but not to PEDF, TNF-α or angiogenesis. Pathologie Biologie 2012;60:e36-e40.
21. Motzer RJ, Hutson TE, McCann L, Deen K, Choueiri TK. Overall survival in renal-cell carcinoma with pazopanib versus sunitinib. N Engl J Med 2014;37:1769-1770.
22. Choi YJ, Oh HR, Choi MR et al. Frameshift mutation of a histone methylation-related gene SETD1B and its regional heterogeneity in gastric and colorectal cancers with high microsatellite instability. Hum Pathol 2014;45:1674-1681.
23. Hakimi AA, Ostrovnaya I, Reva B et al. Adverse outcomes in clear cell renal cell carcinoma with mutations of 3p21 epigenetic regulators BAP1 and SETD2: a report by MSKCC and the KIRC TCGA research network. Clin Cancer Res 2013;19:3259-3267.
24. Manceau G, Marisa L, Boige V et al. PIK3CA muta-

tions predict recurrence in localized microsatellite stable colon cancer. Cancer Med 2015;4:371-382.

25. Paternot S, Colleoni B, Bisteau X, Roger PP. The CDK4/CDK6 inhibitor PD0332991 paradoxically stabilizes activated cyclin D3-CDK4/6 complexes. Cell Cycle (Georgetown, Tex) 2014;13:2879-2888.
26. Shepherd PR, Denny WA. Beta-testing of PI3-kinase inhibitors: is beta better? Cancer Discov 2012;2:393-394.
27. Ni J, Liu Q, X ie S et al. Functional characterization of an isoform-selective inhibitor of PI3K-p110beta as a potential anticancer agent. Cancer Discov 2012;2:425-433