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A-to-I edited miR-411–5p targets MET and promotes TKI response in NSCLC-resistant cells

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Abstract

Non-small cell lung cancer (NSCLC) patients carrying an epidermal growth factor receptor (EGFR) mutation have an initial favorable clinical response to the tyrosine kinase inhibitors (TKIs). Unfortunately, rapid resistance occurs mainly because of genetic alterations, including amplification of the hepatocyte growth factor receptor (MET) and its abnormal activity. The RNA post-transcriptional modifications that contribute to aberrant expression of MET in cancer are largely under-investigated and among them is the adenosine-to-inosine (A-to-I) RNA editing of microRNAs. A reduction of A-to-I editing in position 5 of miR-411–5p has been identified in several cancers, including NSCLC. In this study, thanks to cancer-associated gene expression analysis, we assessed the effect of the edited miR-411–5p on NSCLC cell lines. We found that edited miR-411–5p directly targets MET and negatively affects the mitogen-activated protein kinases (MAPKs) pathway. Considering the predominant role of the MAPKs pathway on TKIs resistance, we generated NSCLC EGFR mutated cell lines resistant to TK inhibitors and evaluated the effect of edited miR-411–5p overexpression. We found that the edited miR-411–5p reduces proliferation and induces apoptosis, promoting EGFR TKIs response in NSCLC-resistant cells.

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AUTHOR CONTRIBUTIONS

GR and MA designed the study. GR, PL, GN, MS, LM, FL, and DDVM coordinated and performed the experiments, and analyzed the corresponding results. GR, PL, and MA wrote the manuscript. All authors contributed to editing the manuscript.

ADDITIONAL INFORMATION

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COMPETING INTERESTS

The authors declare no competing interests.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the United States [1]. Genetic mutations of tyrosine kinase receptors (TKRs), like the epidermal growth factor receptor (EGFR), frequently drive lung cancer development, progression, and drug resistance [2, 3]. Tyrosine kinase inhibitors (TKIs) have become the standard of care for non-small cell lung cancer (NSCLC) patients harboring an EGFR mutation [4, 5]. Patients treated with EGFR TKIs achieve favorable clinical responses initially, but their tumors invariably succumb to drug resistance [6, 7]. One of the most frequent mechanisms of resistance against EGFR TKIs is hepatocyte growth factor receptor (MET) amplification [8]. MET and its downstream signaling pathways (i.e., MAPK, PI3K/AKT) are constitutively activated in several cancers and drive cell survival, proliferation, migration, invasion, and drug resistance [9–11].

Lung cancer research has rapidly evolved from a broad evaluation of the genetic contributors of disease to a more detailed study of the post-transcriptional modifications that may drive cancerous phenotype and drug resistance [12]. In particular, coding and non-coding RNAs can be modified by adenosine deamination into inosine (A-to-I editing) catalyzed by the Adenosine Deaminase Acting on the RNA (ADAR) protein family [13, 14]. A-to-I editing is dysregulated in cancer and involved in drug resistance, including against EGFR TKIs [14, 15]. For example, the altered A-to-I editing of the glycogen synthase kinase 3 (GSK3β) mRNA generates a mis-splicing and induces β-catenin expression, contributing to cancer progression and TKI resistance [16].

Recently, our group identified the dysregulation of A-to-I editing of non-coding RNAs in both tissue and circulation of NSCLC patients [17]. MicroRNAs (miRNAs) are a class of small non-coding RNAs that repress gene expression at the post-transcriptional level [18]. Mature miRNA sequences have a short “seed region” at their 5′ end which binds to complementary regions in the 3′ UTR of target mRNA, inhibiting its translation or promoting its degradation [19]. miRNAs are uniquely deregulated in cancer, acting as tumor suppressors or oncomiRs [20]. Several studies have focused on the regulatory relationship between EGFR, MET, and miRNAs in NSCLC [21–23]. Researchers have observed that miR-130a directly targets MET, activating miR-221/ 222 expression through the c-Jun transcription factor and inducing resistance to TNF-related apoptosis-inducing ligand (TRAIL) in lung adenocarcinoma [24]. Furthermore, MET regulates multiple miRNAs, including miR-221/222, miR-30b/c, miR-103, miR-203, and the miRNA cluster 23a~27a~24–2 [21, 24–26]. The miR-221/222 cluster induces TKI resistance in NSCLC and miR-27a regulates the crosstalk between MET and EGFR, directly targeting both and suppressing Sprouty2 [21]. These studies, among others, have focused on studying miRNA expression and deregulation in cancer. However, few studies to date have investigated miRNA post-transcriptional modifications such as A-to-I editing and their role in cancer and drug resistance [12]. A-to-I editing of miRNAs can alter both their maturation and expression [27]. Furthermore, edits at the seed region can drastically alter the miRNA targetome, thus impacting function [28, 29]. We previously observed that miR-411–5p editing in position 5 of the seed region (ed.miR-411–5p) is reduced in NSCLC patients [17]. Other groups have corroborated these findings in other cancers, including glioblastoma,

stomach, bladder, and breast cancer [30]. However, the biological role of miRNA A-to-I editing in NSCLC remains unknown [30].

In this study, we identified MET as a target for ed.miR-411–5p. Given the role of MET in EGFR TKIs resistance, we investigated the role of non-coding RNA editing on MET signaling, focusing on EGFR TKIs resistance in NSCLC.

RESULTS

A-to-I edited miR-411–5p targets MET and negatively impacts the ERK/MAPK and PI3K/AKT pathways in NSCLC cell lines

In a previous study, we found miR-411–5p hypo-edited in position 5 of the seed region in NSCLC tissues compared with uninvolved lung samples [17]. To further investigate the role of miR-411–5p editing in position 5 in NSCLC, we generated a custom-designed mimic of the edited form of ed.miR-411–5p, where the inosine in position 5 (I) was replaced by guanosine (G) (Horizon). The replacement of I-to-G is commonly used to mimic the inosine functions experimentally [31]. We then overexpressed the ed.miR-411–5p by transfection in two NSCLC cell lines (H1299 and H520) (Fig. 1a left).

To assess the edited miR-411–5p transfection, we purchased a probe custom designed to specifically detect the edited version of miR-411–5p exposing an A to I (G) site at position 5 (ThermoFisher Scientific) (Supplementary Fig. 3a). See the Materials and Methods section for more details.

A NanoString nCounter PanCancer Pathways Panel was then performed to identify dysregulated genes associated with 13 different cancer-associated pathways. We found 30 dysregulated genes in H520 and 83 genes in H1299 ($P < 0.01$) (Fig. 1a right; Supplementary Table 1; see Materials and Methods section for more details). Functional enrichment analysis was conducted to detect the most relevant pathways involved in lung cancer development and progression. We used the Ingenuity Pathway Analysis® (IPA®) software to accomplish this and found several pathways associated with cell signaling and drug resistance in both of the cell lines. We focused on the ERK/ MAPK and PI3K/AKT pathways which were significantly enriched in both H1299 and H520 cell lines by the ed.miR-411–5p, showing a negative Z score in the H1299 (Fig. 1b; Supplementary Table 2).

To identify possible direct targets of ed.miR-411–5p, we considered the downregulated genes common to both cell lines (Fig. 1a red circle) with unique predicted binding sites for the ed.miR-411–5p (Supplementary Table 1; Supplementary Fig. 2d). Among them, we elected to study MET for its preeminent role in the ERK/MAPK and PI3K/AKT pathways, NSCLC biology, and drug resistance [8, 9, 32]. We also predicted, in silico, that the MET mRNA 3′ untranslated region (3′UTR) would be targeted by ed.miR-411–5p (Fig. 1c) but not miR-411–5p wild type (WT). We preliminarily assessed by western blot the effect of ed.miR-411–5p on the MET protein compared to the scramble (Scr) control and miR-411–5p WT in two NSCLC cell lines (H1299 and A549). We found that only the ed.miR-411–5p but not the miR-411–5p WT represses MET protein levels (Fig. 1d, Supplementary Fig. 3b, c). See the Material and Methods section for more details.

To further validate the MET mRNA direct targeting by ed.miR-411–5p, we cloned a portion of the MET mRNA 3′UTR containing the predicted binding site for ed.miR-411–5p into a psicheck2 vector (Promega Corporation - Madison, Wisconsin, USA) downstream of the Renilla open reading frame (ORF). The MET-3′UTR luciferase reporter plasmid was co-transfected with the ed.miR-411–5p and a Scr control. The ed.miR-411–5p significantly repressed the luciferase activity of the reporter vector when compared to the Scr control, demonstrating the direct targeting of MET (Fig. 1e - left). We then mutated the predicted binding region on MET 3′UTR as a control, finding a significant increment of the luciferase activity (Fig. 1e - right).

Finally, to evaluate the potential function of ed.miR-411–5p (pos. 5), we performed a genome-wide unbiased consensus prediction analysis on canonical target by employing the isoTar tool [33], followed by a pathway enrichment analysis (see the Materials and Methods section for more details). Globally, we found 1431 predicted targets for the ed.miR-411–5p; among them, 65 (TOP) targets have been predicted by at least 3 out of 5 prediction tools we employed for the analysis (PITA, RNAhybrid, TargetScan, miRanda, miRmap) [33]. Leveraging these data, we also implemented an Ingenuity® Pathway Analysis (IPA®) software analysis predicting 90 biological pathways (-log(p-value) >1.301, corresponding to a *p*-value<0,05) impacted by the ed.miR-411–5p. We then compared these pathways with the ones we previously found in NSCLC cells (Fig. 1b and Supplementary Table 2) which resulted in 54 overlapped pathways. Among them, two TKR pathways (PDGF and VEGF) were significantly enriched by ed.miR-411–5p (Supplementary Table 3).

Edited miR-411–5p reduces c-Fos expression and AP1 activity in NSCLC

c-Met regulates AP1 activity and c-Fos expression through the ERK pathway (Fig. 2a) [34]. To validate the predicted negative impact of ed.miR-411–5p in this pathway presented in Fig. 1b, we first evaluated MET/MAPK/ERK signaling by western blot after transfection with the ed.miR-411–5p and Scr control and found a downregulation of p-ERK and p-Elk levels in H1299 after transfection with ed.miR-411–5p (Fig. 2b, Supplementary Fig. 2c, Supplementary Fig. 3d). To further validate this effect, we used a Serum Response Element (SRE) luciferase reporter vector (BPS Bioscience - San Diego, California, USA) to monitor the MAPK/ ERK pathway transcriptional activity. After transfection with ed.miR-411–5p, the luciferase activity in A549 cells was significantly reduced compared to the Scr control (Fig. 2c). We also evaluated p-Elk and total Elk levels in A549 cells after transfection with the ed.miR-411–5p or Scr control and found a decrease in both p-Elk and total Elk as a consequence of ed.miR-411–5p overexpression (Fig. 2d, Supplementary Fig. 2c, Supplementary Fig. 3e). Due to the presence of SRE elements on the c-Fos promoter and transcriptional activation of c-Fos by Elk [35, 36], we next evaluated the effect of ed.miR-411–5p on c-Fos expression by assessing c-Fos mRNA levels by qRT-PCR (Fig. 2e) and protein levels by western blot in NSCLC cell lines (Fig. 2f and Supplementary Fig. 1a – top, Supplementary Fig. 3i). We found that overexpression of ed.miR-411–5p but not the miR-411–5p WT reduces c-Fos expression (Supplementary Fig. 1a – bottom, Supplementary Fig. 2d, Supplementary Fig. 3b). Intriguingly, we also predicted a possible ed.miR-411–5p binding site in the c-Fos mRNA 3′UTR (Supplementary Fig. 1b; Supplementary Fig. 2d). The c-Fos 3′UTR containing the ed.miR-411–5p binding site was cloned into a psicheck2

vector (Promega Corporation) downstream of the Renilla ORF to investigate whether c-Fos is a direct target of ed.miR-411–5p. A luciferase assay was performed co-transfecting the ed.miR-411–5p, miR-222 (positive control), and Scr control along with the aforementioned luciferase plasmid. We selected miR-222 as a positive control because there is a known miR-222 binding site present in the portion of the c-Fos 3′UTR that we cloned in the psicheck2 vector [37]. As shown in Supplementary Fig. 1c, we did not find any direct targeting of the c-Fos 3′UTR by the ed.miR-411–5p.

We also wanted to test the role of ed.miR-411–5p on the AP1 pathway. AP1 activity is greatly dependent on ERK activity [38]. For this objective, we employed a luciferase plasmid in which the responsive element for the AP1 complex was cloned as a promoter element [39]. As shown in Fig. 2g, the luciferase activity was significantly reduced after transfection of ed.miR-411–5p. An ERK inhibitor (Sigma) (3 μM) was used as a positive control in this set of experiments. Considering the role of AP1 on c-Myc and c-Jun transcription factors [40], we also assessed the protein and mRNA levels of c-Jun and c-Myc after ed.miR-411–5p over-expression in H1299 cells. We found a reduced expression of c-Jun and c-Myc protein and mRNA (Fig. 2h, i, Supplementary Fig. 3a left). These results further confirm the inhibiting role of ed.miR-411–5p on the ERK-AP1 pathway.

Edited miR-411–5p increases TKIs sensitivity in NSCLC

Overexpression of c-Met is a common mechanism of resistance to EGFR TKIs [8]. To test the effect of ed.miR-411–5p on TKI resistance, we rendered an EGFR mutated and TKIs sensitive NSCLC cell line (HCC827) resistant to gefitinib by treating the cells with two different concentrations of gefitinib (2.5 μM and 5 μM) for a minimum of four weeks. We generated two cell lines with different degrees of resistance to gefitinib which we named HCC827R mild (2.5 μM) and HCC827R (5 μM) and used these cell lines for our subsequent experiments (Fig. 3a). It has been reported that HCC827 can develop resistance to TKIs through MET amplification [41, 42]. We assessed MET levels in the HCC827R mild and HCC827R cell lines by western blot and qRT-PCR, finding a progressive increase in MET protein expression and mRNA levels in HCC827R mild and HCC827R (Fig. 3b, c). We first evaluated the effect of the ed.miR-411–5p on MET, p-Akt, and p-ERK levels in HCC827R mild by western blot. We found that the ed.miR-411–5p reduces MET levels and its pathway in HCC827R mild compared to the Scr control (Fig. 3d, Supplementary Fig. 3f). We further evaluated the effect of ed.miR-411–5p on cell proliferation in HCC827R mild cells treated with gefitinib (5 μM). The ed.miR-411–5p significantly reduced HCC827R mild cell growth when combined with gefitinib compared to the vehicle treatment (Fig. 3e; Supplementary Fig. 1d). We then tested the effect of ed.miR-411–5p on MET, p-Akt, and p-ERK levels in HCC827R by western blot. As reported in Fig. 3b, the HCC827R cells express higher levels of MET protein when compared to the HCC827R mild. In HCC827R cells with higher MET upregulation, overexpression of ed.miR-411–5p fails to reduce MET and p-Akt protein levels while still reducing p-ERK levels (Fig. 3f, Supplementary Fig. 3g). Furthermore, once MET expression is upregulated, the ed.miR-411–5p overexpression in combination with gefitinib (5 μM) cannot repress HCC827R proliferation (Fig. 3g; Supplementary Fig. 1e - top), confirming a link between the ed.miR-411–5p and MET signaling on TKIs resistance in HCC827R cells. In addition to gefitinib, HCC827R cells were also resistant to the third-

generation EGFR TKI osimertinib [42]. Once the ed.miR-411–5p was overexpressed in combination with osimertinib (5 μM), it was able to induce weak but significant repression in HCC827R cell growth (Fig. 3g; Supplementary Fig. 1e - top). Considering the effect of ed.miR-411–5p on ERK phosphorylation in HCC827R cells independent from MET expression (Fig. 3f), we hypothesized a MET-independent effect of the ed.miR-411–5p on ERK signaling. To further investigate this hypothesis, we generated a second NSCLC cell line (PC9) that is resistant to EGFR TKI (gefitinib - 5 μM) (PC9R) (Fig. 3h). PC9 cells carry an EGFR mutation and are sensitive to EGFR TKIs [42]. It has been reported that PC9 cells acquire EGFR TKIs resistance in a MET-independent manner [42]. We evaluated MET expression in PC9R by western blot and qRT-PCR and did not detect MET amplification in this TKIs-resistant NSCLC cell line (Fig. 3i). We then evaluated the effect of the ed.miR-411–5p in the PC9R cell line. We found that MET and p-ERKs levels were reduced when ed.miR-411–5p was overexpressed (Fig. 3j, Supplementary Fig. 3h). To determine the biological effect of ed.miR-411–5p in the PC9R cell line, we performed a colony assay overexpressing the ed.miR-411–5p and treating the PC9R with EGFR TKIs (gefitinib or osimertinib - 5 μM). In combination with gefitinib or osimertinib, ed.miR-411–5p reduced cell proliferation in PC9R cells, which are EGFR TKIs resistant but not MET amplified (Fig. 3k; Supplementary Fig. 1e - bottom).

To further assess the role of the ed.miR-411–5p in TKI response, we performed an Annexin-V5 assay in HCC827R and PC9R cell lines after ed.miR-411–5p overexpression and EGFR TKI treatment (gefitinib or osimertinib - 5 μM). The ed.miR-411–5p significantly induced apoptosis in both HCC827R and PC9R cells when combined with an EGFR TKI (Fig. 4a, b).

Finally, we also evaluated apoptosis by caspase 3/7 assay after transfecting A549 NSCLC cells with Scr, ed.miR-411–5p, or miR-411–5p WT and treating transfectants with gefitinib (5 μM). Parental A549 cells express mutated KRAS (G12S) but WT EGFR, and are resistant to EGFR TKIs [43–45]. In A549 cells, the ed.miR-411–5p induced caspase 3/7 activity in combination with gefitinib. In contrast, the miR-411–5p WT did not promote gefitinib response in the A549 cell line (Supplementary Fig. 2a). We further evaluated apoptosis in A549 cells by Annexin-V5 after Scr or ed.miR-411–5p transfection and gefitinib (5 μM) treatment. The ed.miR-411–5p significantly induced apoptosis when combined with gefitinib in A549 cells (Supplementary Fig. 2b).

DISCUSSION

EGFR mutations occur in 10–20% of NSCLC cases in Caucasians and up to 50% of NSCLC cases in Asians [46, 47]. EGFR TKIs have become the first-line therapy used to treat NSCLC patients harboring a somatic mutation in EGFR [4]. Unfortunately, despite a high objective response rate, many patients on EGFR TKIs treatment eventually develop resistance and progress after a median of 10–14 months [48]. Recently, investigators have focused on elucidating the molecular drivers and mechanisms of EGFR TKIs resistance. One identified mechanism is through the amplification of the receptor tyrosine kinase MET which overrides the EGFR inhibitor’s action, inducing proliferation and resistance in up to 22% of cases of EGFR TKI-resistant NSCLC [6]. The molecular mechanisms of

MET-induced EGFR TKIs resistance in cancer have been widely investigated, including the role of MET-dependent non-coding RNAs. In particular, the dysregulated expression of miRNAs, like miR-221/222 and miR-130a, plays an essential role in driving MET-induced drug resistance [21, 24–26, 49]. Here, we focused beyond the dysregulated expression of miRNAs in cancer, examining altered post-transcriptional modification of miRNA, such as A-to-I editing in NSCLC.

In our previous work, we observed that A-to-I editing of miR-411–5p in position 5 is reduced in tissues and circulation of NSCLC patients [17]. It is reported that miR-411–5p can be edited by ADAR2 [50, 51]. We found a reduction of ADAR2 in lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC) tissue and normal tissue (483 LUAD vs 59 normal; 486 LUSC vs 50 normal) from TCGA RNA sequencing data. In the same comparison, we found that ADAR1 and ADAR3 expression were unchanged (Supplementary Fig. 4). This finding suggests that reduced A-to-I editing of miR-411–5p that we previously observed in NSCLC [17] could be dependent on ADAR2 changes in NSCLC tissues. Intriguingly, the reduction of A-to-I editing within the seed region (position 5) of the miR-411–5p has also been detected in other cancers [30]. A-to-I editing within the miRNAs' seed region alters its function by inducing a targetome shift and generating *de facto* a new miRNA [28, 29].

We decided to investigate the role of ed.miR-411–5p in NSCLC by analyzing 770 genes from 13 cancer-associated canonical pathways. We found that ed.miR-411–5p impacts TKR pathways, inhibits the extracellular signal-regulated kinases (ERKs), and targets MET directly. We also demonstrated that only the ed.miR-411–5p in position 5, and not the miR-411–5p WT, directly targets MET and reduces MET protein levels. We validated that ed.miR-411–5p represses ERK signaling activity, which is downstream of MET activation [34]. We also found that ed.miR-411–5p specifically reduces the expression of c-Fos, suppressing the phosphorylation of the ERKs and Elk-1 pathway. Additionally, we evaluated the effect of ed.miR-411–5p on the AP1 transcription factor activity in NSCLC which also depends on the MET and ERKs pathways [34, 38, 52]. We found that ed.miR-411–5p represses the AP1 transcriptional activity and reduces downstream c-Myc and c-Jun levels in NSCLC cells.

Considering the prominent role of MET and ERK pathways in NSCLC drug resistance [8], we decided to investigate the effect of ed.miR-411–5p on EGFR TKIs resistance by generating two EGFR mutated NSCLC cell lines that have developed EGFR TKIs resistance (gefitinib and osimertinib). We chose two NSCLC cell lines that develop resistance to EGFR TKIs differently: the HCC827 cell line generally develops resistance through MET amplification, while the PC9 cell line develops resistance in a MET-independent manner [41, 42]. Considering the role of ed.miR-411–5p on MET, we wanted to assess the effect of its overexpression on HCC827R mild and HCC827R, which were made resistant to different concentrations of gefitinib. The HCC827R mild cells overexpress MET compared to the HCC827 parental cells and are resistant to ≤2.5 μM of gefitinib or osimertinib. The HCC827R cells express the highest level of MET compared to the HCC827 parental and HCC827R mild cells and are resistant to ≤5 μM of gefitinib or osimertinib. We evaluated the expression levels of edited and WT miR-411–5p in all of the cell lines we used for our

experiments, particularly for the HCC827, HCC827R-mild, and HCC827R. In all of the cell lines, the levels of both WT and edited miR-411–5p were undetected or very low (Ct >33, data not shown), suggesting there is not a significant correlation between MET amplification and miR-411–5p expression. We overexpressed ed.miR-411–5p in both HCC827R mild and HCC827R cells and assessed cell proliferation after treatment with gefitinib or osimertinib 5 μM. We found that ed.miR-411–5p failed to repress MET and did not induce a significant response to gefitinib in HCC827R cells. This observation demonstrates that MET overexpression in HCC827R cells affects the ability of ed.miR-411–5p to induce an EGFR TKIs response, proving that the effect of ed.miR-411–5p depends, at least in part, on MET targeting. We hypothesized that the lack of MET targeting in HCC827R by ed.miR-411–5p could depend on post-transcriptional modifications of MET mRNA which can render the transcript more stable [53]. Interestingly, although ed.miR-411–5p does not effectively target MET in HCC827R cells, p-ERK levels were still suppressed despite the fact we consistently observed an upregulation of total ERK. These findings suggest a role for ed.miR-411–5p in the ERK pathway in a MET-independent manner. This observation is supported by the inhibition of Elk-1 activity and the EGFR TKI response induced by ed.miR-411–5p in the A549 NSCLC cell line. A549 cells are KRAS mutated (G12S), and the ERK pathway responds only minimally to upstream inhibition of MET or EGFR [45, 54].

To further investigate this, we also evaluated whether ed.miR-411–5p overexpression induces EGFR TKIs response in PC9R cells that developed TKIs resistance in a MET-independent manner. The ed.miR-411–5p suppressed MET and p-ERK levels in PC9R cells and induced a strong EGFR TKIs response to both gefitinib and osimertinib. The response of these cells to EGFR TKIs treatment is minimally explained by MET repression because PC9 cells do not develop resistance by amplifying MET. We also found that ed.miR-411–5p significantly induced apoptosis in HCC827R and PC9R NSCLC cell lines.

These results shed light on the potential importance of miRNA post-transcriptional modifications in drug response in cancer. The A-to-I editing of miR-411–5p in position 5 is reduced in cancers, including NSCLC (edited/wt ratio), suggesting a role in TKIs response rather than TKIs resistance. The A-to-I editing modification within the seed region induces a targetome shift of the edited miRNA compared to the WT. We found that ed.miR-411–5p directly targets MET and represses the MAPK pathway. We also demonstrated that ed.miR-411–5p induces EGFR TKIs sensitivity in EGFR TKI-resistant cell lines only partially dependent on MET repression. Indeed, our results suggest that other potential targets of the ed.miR-411–5p are involved in the drug response we observed in TKI-resistant NSCLC cells. In particular, the dysregulation of two ed.miR-411–5p predicted targets, IGF1 and NTRK2 genes, involved in the STAT3 and PTEN pathways needs to be further investigated in this respect [55–57]. Finally, more comprehensive studies are needed to further define the role of ed.miR-411–5p in cancer as a potential biomarker or therapeutic target in TKI-resistant NSCLC.

MATERIALS AND METHODS

Cell culture, transfection, and chemicals

All cell lines (H520, A549, H1299, PC9, HCC827, Hek-293) were grown in RPMI with 10% FBS, L-glutamine, and antibiotics (Sigma - Burlington, MA, United States). Transfections were performed using Lipofectamine 3000 (ThermoFisher - Waltham, Massachusetts, USA) as suggested by the manufacturer. All cell lines were transfected at 60% confluence in OPTIMEM® (ThermoFisher) without antibiotics and then transfected with 100 nM of custom-made ed.miR-411–5p oligonucleotides, miR-411–5p WT or negative control (Horizon Cambridge, United Kingdom) for the indicated time.

The sequences of edited miR-411–5p are:

Active:

5′ P.U.A.G.U.G.G.A.C.C.G.U.A.U.A.G.C.G.U.A.C.G 3′

Passenger:

5′ U.A.C.G.C.U.A.U.A.C.G.G.U.C.C.A.C.U.A.U.U 3′

Western blot analysis

H520, A549, H1299, PC9, HCC827, HCC827R mild, HCC827R, and PC9R cells were seeded and grown in RPMI (Sigma) with 10% FBS in 6 well plates for 24 h before the transfection. Then, 48 h after transfection, cells were washed in PBS 1X and collected for RNA and protein extraction. Protein extraction and western blot were performed as previously described [58].

Antibody used for western blot analysis

Vinculin (ab18058) and GAPDH (ab8245) antibodies were obtained from Abcam (Cambridge, UK). p-Akt (#9275 S), p-ERK1/2 (#9101 S), p-Elk (#9181 S), Akt (#9272 S), ERK1/2 (#4696 S), Elk (#9182 S), c-Met (#4560 S), c-Jun (#9165), and c-Myc (#18583) antibodies were obtained from Cell Signaling Technologies (Danvers, Massachusetts, USA). The c-Fos (#sc-271243) antibody was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA).

RNA extraction

Total RNA was extracted using the RNA Cleanup and Concentration Kit (Norgen - Thorold, ON, Canada) following the kit protocol.

Real-time qRT-PCR

All the qRT-PCRs were performed with Taqman® technology as previously described [59]. We used the custom TaqMan Advanced miRNA assay (PN CCU001M) and inventoried TaqMan Advanced miRNA assay (both are from ThermoFisher Scientific) to check the expression of edited miR-411–5p and WT miR-411–5p, respectively. As shown in Supplementary Fig. 3b, c, the probe we designed specifically detects the edited version

of miR-411–5p and not the transfected miR-411–5p WT. On the contrary, the probe for detecting miR-411–5p WT was unable to distinguish the two transfected miRNAs (WT and edited).

Luciferase assay

To generate c-Met and c-Fos luciferase reporter constructs, a part of the 3′ UTRs was amplified by PCR and cloned downstream of Renilla CDS in the psicheck2 plasmid (Promega). Mutations were introduced into the miRNA-binding sites using the QuikChange Mutagenesis Kit (Stratagene - La Jolla, California, USA). HEK-293 cells were transfected with the Psicheck2-met-3′ UTR, Psicheck2-met-3′UTR-MUT, or Psicheck2-Fos-3′UTR plasmids and miRs using Lipofectamine 3000 (Thermo Fisher). After 24 h, cells were lysed and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions.

The primers used for the cloning were as follows:

c-Fos FW:

CCGCTCGAGCGGGGTGCATTACAGAGAGGAGAAA

c-Fos RV:

AAGGAAAAAAGCGGCCGCAAAAGGAAAACCTCAACAATGCATGATCAGTAAC

MET FW:

CCGCTCGAGCGGCTATGTCCATGTGAACGCTACT

MET RV:

AAGGAAAAAAGCGGCCGCAAAAGGAAAATATGATCACACCACTGCACTAC

Growth density assay

The cells were harvested 6 h after transfection by trypsinization, and 5×10^5 cells were seeded into 6 well plates in triplicate. The cells were treated with DMSO, gefitinib, or osimertinib. After incubation, they were then fixed and stained with Crystal violet solution (Sigma). The images were obtained for density analysis with the ChemiDoc™ MP Imaging System (Bio-Rad) and quantified by ImageJ.

Apoptosis

Apoptosis was assessed using the Annexin V-FITC apoptosis detection kit (ThermoFisher) followed by flow-cytometric analysis. PC9R and HCC827R cells were transfected and, after 24 h, treated with DMSO, gefitinib, or osimertinib. After incubation, cells were washed with cold PBS and removed from the plates by trypsinization. The resuspended cells were washed with cold PBS and stained with FITC-conjugated annexin-V antibody according to the manufacturer's instructions (ThermoFisher). Samples were allowed to stand for 15

min in the dark and immediately analyzed using the FACS-Calibur flow cytometer (BD Bioscience - Franklin Lakes, New Jersey, USA).

Differential expression analysis after edited miR-411–5p transfections

We performed differential expression analysis of 770 genes of the nCounter PanCancer Pathways Panel in biological triplicates using NanoString Technology®. Normalization was performed using the nSolver Analysis Software (Version 4.0; NanoString Technologies), applying the geometric mean of the negative controls for negative background subtraction; the positive controls for the technical normalization; and the housekeeping genes for the biological normalization in all samples, as recommended by NanoString. *P* values were calculated using the *limma* package (v3.48.3) from the Bioconductor R project. *P* values were used to rank all genes, retaining those under a significant threshold of 0.01 and with a geometric mean expression of >20 counts (in at least one condition) after negative background subtraction and a |linear fold-change | > 1.25.

Edited miR-411–5p targeting prediction for 3′ UTR genes

We predicted canonical and non-canonical binding sites on 3′ UTR sequences (UCSC.hg19) of MET, c-Fos, TIAM1, and NOTCH2 genes by using RNAhybrid (v2.1.2) predictor tool [60], considering a $\Delta G \leq 20$ kcal/mol (free energy). In particular, for non-canonical binding sites, we allowed G:U pairings in the seed region and at least six bases of pairings with the 5′ end of the miRNA sequences after the first or second nucleotide of the miRNA.

Pathway enrichment analysis after edited miR-411–5p transfection in H1299 and H520 cell lines

Using Ingenuity® Pathway Analysis (IPA®) software (v01–20-04), we performed functional enrichment analysis on the retained set of differentially expressed genes (mentioned above). Settings used included experimentally observed data for the human species.

Genome-wide and unbiased prediction analysis for edited miR-411–5p and pathway enrichment analysis.

To perform a genome-wide and unbiased prediction analysis for the edited miR-411–5p, we employed a canonical prediction analysis with a consensus of 3 out of five predictors (PITA, RNAhybrid, TargetScan, miRanda, miRmap) by using isoTar (v1.2.1) [33]. We then performed the pathway enrichment analysis via the IPA software, as described above.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study have been submitted to the GEO repository (accession # GSE221334).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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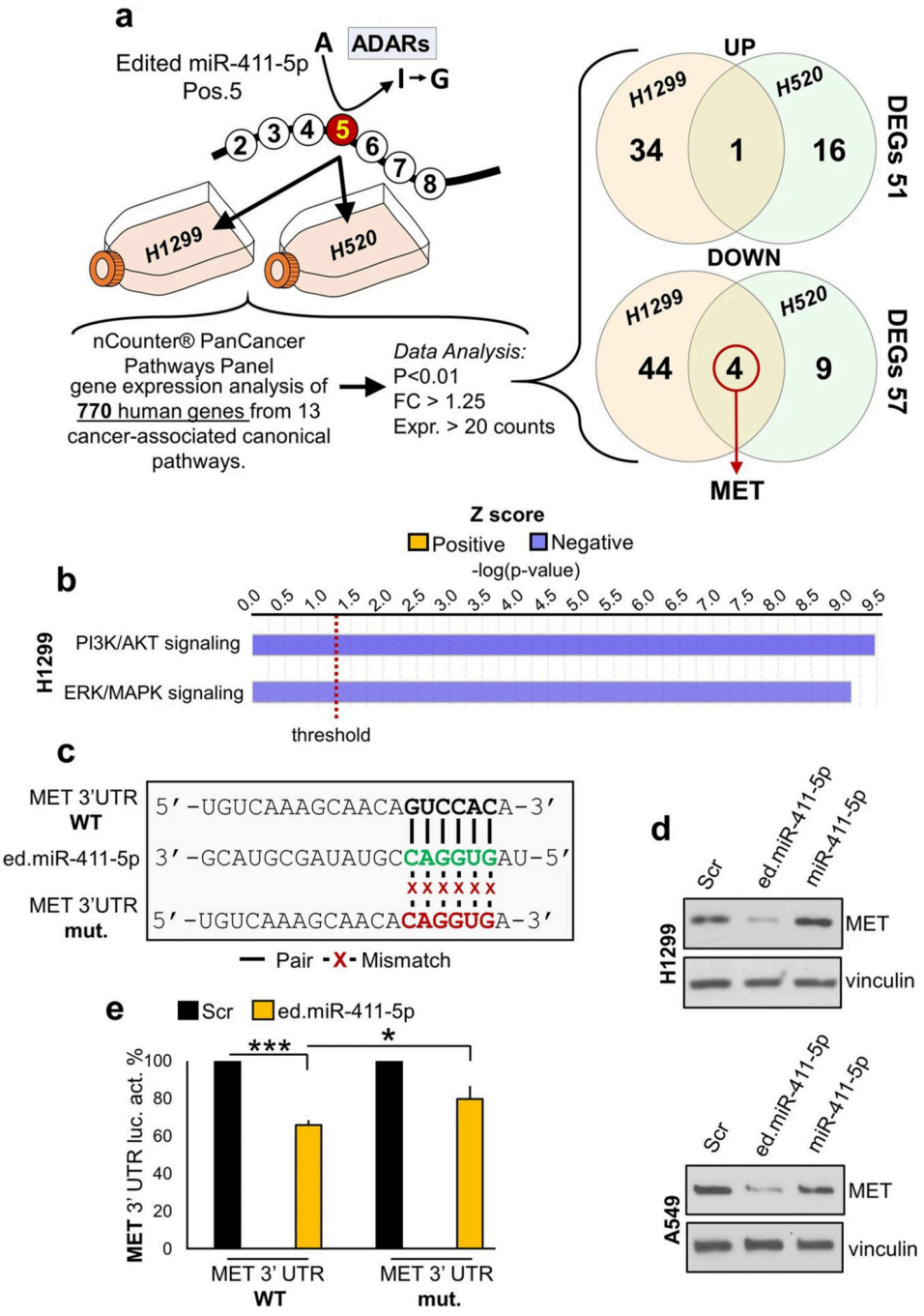

Fig. 1. Edited miR-411–5p targets MET and impacts the ERK/MAPK and PI3K/AKT pathways.

a Workflow for nCounter PanCancer Pathways Panel® analysis of ed.miR-411–5p overexpression in NSCLC cell lines (left). Venn diagrams (right) show deregulated genes (up and down) detected via the nCounter PanCancer Pathways Panel® in both H1299 and H520 cell lines overexpressed with ed.miR-411–5p. **b** IPA® analysis on H1299 after ed.miR-411–5p transfection shows its negative impact on ERK/MAPK and PI3K/AKT pathways. **c** The predicted alignment of the seed regions of ed.miR-411–5p (green) with MET 3′UTR (black). mfe is −23.2 kcal/mol, *p*-val0.983502. The site of targeted

mutagenesis is indicated in red. **d** Western blots show the expression of endogenous MET in H1299 and A549 after transfection with Scr, ed.miR-411–5p or miR-411–5p wild type. **e** Luciferase activity assay of psicheck2-MET 3′UTR luciferase reporter plasmids containing WT or mutated (mut.) MET 3′UTRs transfected with Scr or ed.miR-411–5p. The statistical significance of the results was determined using two-tailed Student's *t*-test. *p*-value *<0.05 **<0.01 ***<0.001. Nonsignificant *p*-values were not reported. All of the western blots were done in a minimum of a biological triplicate.

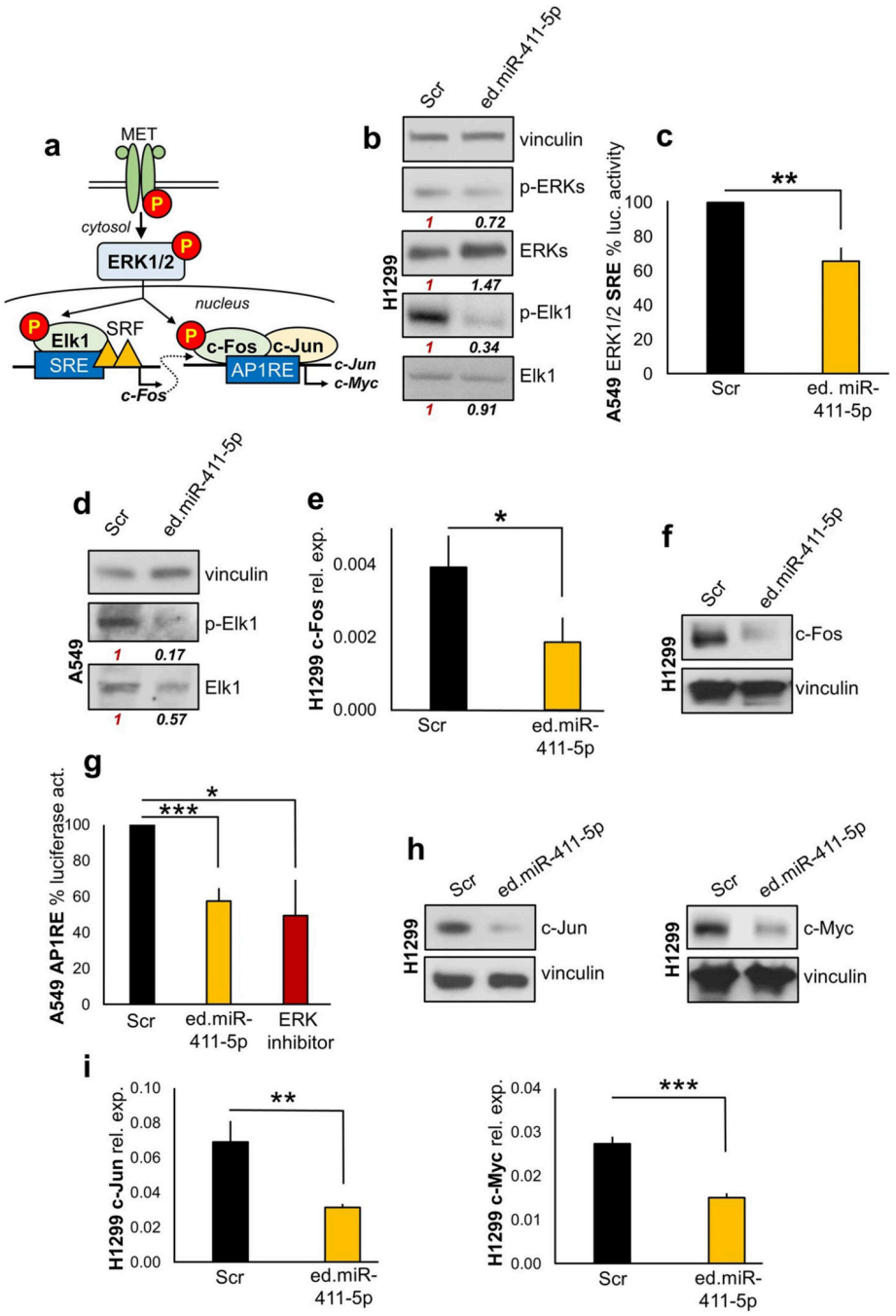

Fig. 2. Edited miR-411–5p reduces c-Fos expression and AP1 activity.

a Schema of MET signaling pathway. **b** Western blots showing the phosphorylation of ERKs and Elk in H1299 after transfection with Scr or ed.miR-411–5p. **c** SRE-luciferase reporter plasmid assay graph in A549 cells transfected with Scr or ed.miR-411–5p. **d** Western blots showing the phosphorylation of Elk in A549 after the transfection of Scr or ed.miR-411–5p. **e** H1299 c-Fos mRNA relative expression (qRT-PCR) after ed.miR-411–5p or Scr transfection. GAPDH was used for normalization. **f** Western blots showing c-Fos levels in H1299 transfected with Scr or ed.miR-411–5p. **g** AP1-luciferase reporter

plasmid assay graph of A549 transfected with Scr, ed.miR-411–5p or treated with ERKs inhibitor (3 μM) as control. **h** Western blots showing c-Jun (left) and c-Myc (right) levels in H1299 transfected with Scr or ed.miR-411–5p. **i** H1299 c-Jun (left) and c-Myc (right) mRNA relative expression (qRT-PCR) after ed.miR-411–5p or Scr transfection. β-actin and GAPDH, respectively, were used for normalization. The statistical significance of the results was determined using two-tailed Student's *t*-test. *p*-value *<0.05 **<0.01 ***<0.001. Nonsignificant *p*-values were not reported. All of the western blots were done in a minimum of a biological triplicate.

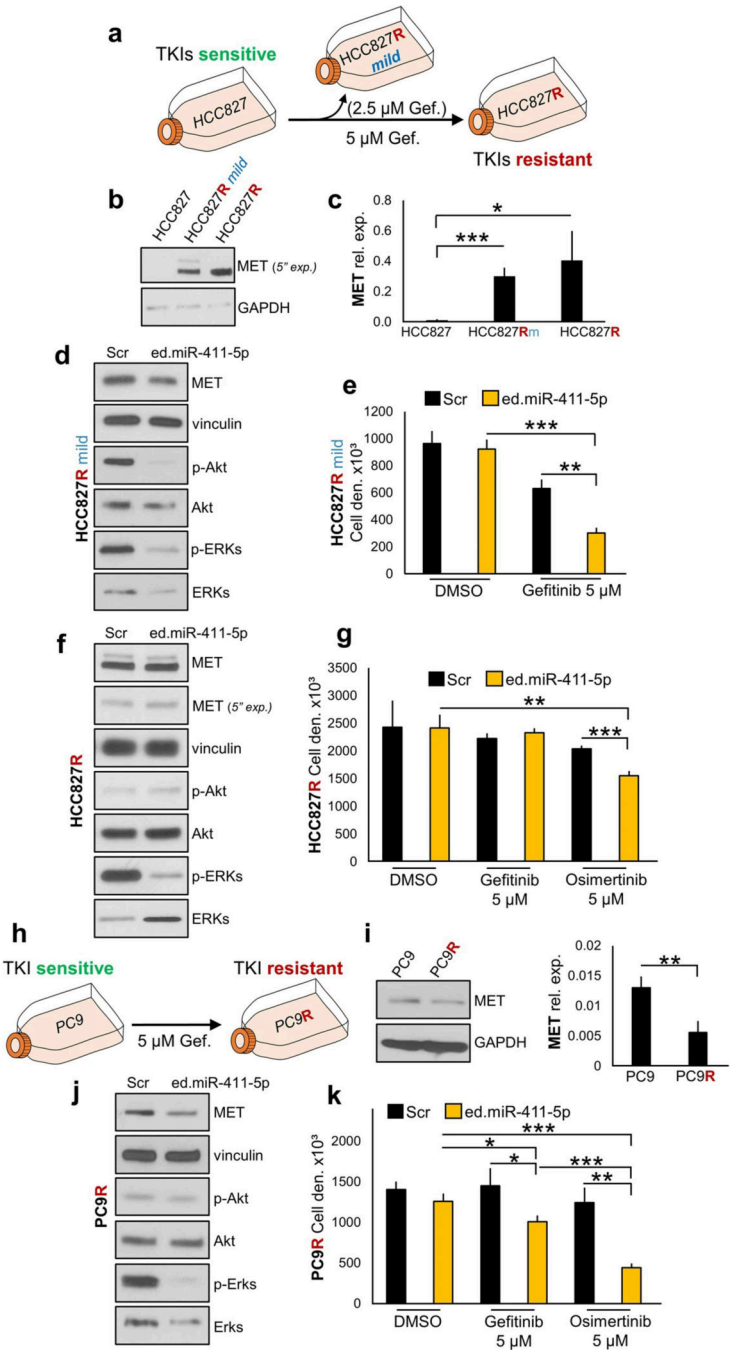

Fig. 3. Edited miR-411–5p reduces cell proliferation in response to TKIs.

a Schema of HCC827R mild and HCC827R EGFR TKIs resistant cell lines generation. **b** Western blots showing MET expression in HCC827 parental, HCC827R mild, and HCC827R cell lines. **c** MET mRNA relative expression (qRT-PCR) in HCC827 parental, HCC827R mild, and HCC827R cell lines. GAPDH was used for normalization. **d** Western blots showing MET, p-AKT, and p-ERKs levels in HCC827R mild cells transfected with Scr or ed.miR-411–5p. **e** Graph of the cell proliferation density of HCC827R mild transfected with Scr or ed.miR-411–5p and treated for 72 h with gefitinib (5 μM). **f** Western blots

showing MET, p-AKT, and p-ERKs levels in HCC827R cells transfected with Scr or ed.miR-411–5p. **g** Graph of the cell proliferation density of HCC827R cells transfected with Scr or ed.miR-411–5p and treated for 72 h with gefitinib (5 μM) or osimertinib (5 μM). **h** Schema of PC9R EGFR TKIs resistant cells generation. **i** Western blots showing MET protein (left) and MET mRNA expression (qRT-PCR, right) in PC9 parental and PC9R cell lines. GAPDH was used for normalization. **j** Western blots showing MET, p-AKT, and p-ERKs levels in PC9R cells transfected with Scr or ed.miR-411–5p. **k** Graph of the cell proliferation density of PC9R transfected with Scr and ed.miR-411–5p and treated for 72 h with gefitinib (5 μM) or osimertinib (5 μM). The statistical significance of the results was determined using two-tailed Student's *t*-test. *p*-value *<0.05 **<0.01 ***<0.001. Nonsignificant p-values were not reported. All of the western blots were done in a minimum of a biological triplicate.

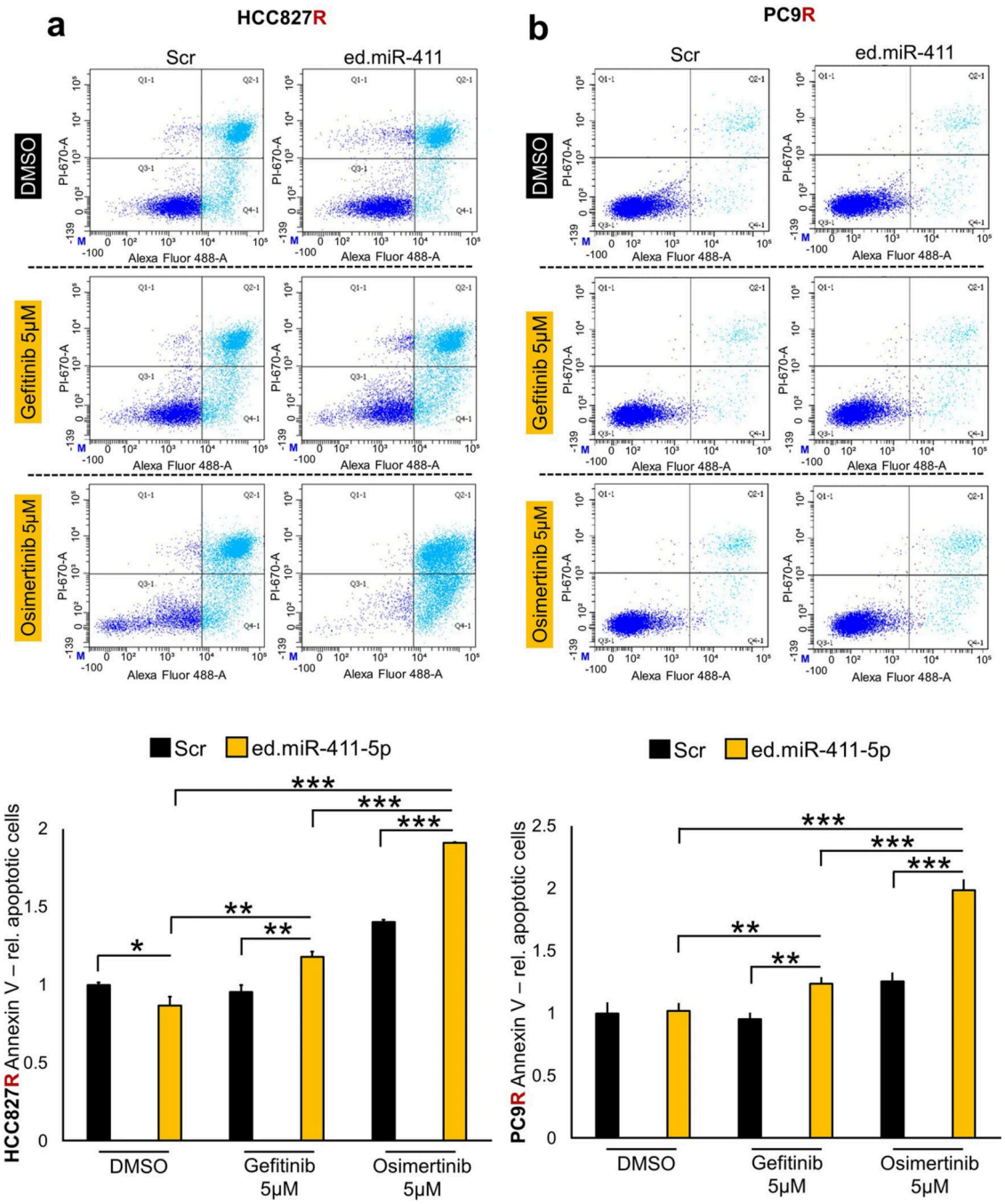

Fig. 4. Edited miR-411–5p induces apoptosis in response to TKIs.

a, b Representative plots of Annexin V5 assay on HCC827R and PC9R cells transfected with Scr or ed.miR-411–5p and treated with DMSO, gefitinib (5 μM), or osimertinib (5 μM) for 72 h (top); bar graph representing the relative apoptotic cells (bottom). Data are all relative to DMSO with Scr. The statistical significance of the results was determined using two-tailed Student's *t*-test. *p*-value *<0.05 **<0.01 ***<0.001. Nonsignificant p-values were not reported.