SETD2 and miR-21 as therapeutic targets for NUT midline carcinoma

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Abstract

Background:

Nuclear protein in testis (NUT) midline carcinoma (NMC) is a rare and highly aggressive tumor with the bromodomain containing 4 (*BRD4*)-*NUT* (*NUTM1*) gene fusion. BRD4 is a member of the bromodomain and extra-terminal domain (BET) family of proteins, and BET inhibitors have been investigated in NMC clinical trials. However, few targeted therapies are available for NMC, and novel therapeutic targets remain to be determined. We determined the role of two epigenetic regulators as possible therapeutic targets for NMC.

Methods:

We performed next-generation sequencing (NGS) in NMC cell lines (HCC2429 and Ty82). H3K36me3 expression was studied using western blotting. The efficacy of AZD1775, a WEE1 inhibitor, was evaluated using the MTS and γH2AX assays. We established an NMC cell line that was resistant to BET inhibitors. The sensitivity of the cells resistant to AZD1775 was analyzed using the MTS assay. RNA sequencing was performed to determine miRNA expression levels. TaqMan miRNA assays were used to analyze miR-21 expression. The efficacy of the miR-21 inhibitor was evaluated using the MTS assay. We established a digital PCR (dPCR) assay to detect *NUT* gene rearrangements to identify patients with NMC. Using NGS, a patient with NMC was identified with the *SETD2* mutation.

Results:

SETD2 mutation (p.Ser2382fs) was determined in NMC cells, in which H3K36me3 expression was depleted, indicating SETD2 loss. NMC cells were sensitive to the WEE1 inhibitor, AZD1775 in cancer cells with SETD2 deficiency. γH2AX expression was increased in NMC cells treated with AZD1775. The efficacy of the combination of AZD1775 and JQ-1 was additive. We established NMC cells that were resistant to BET inhibitors. The resistant cells were also sensitive to AZD1775. miRNA analysis revealed increased miR-21 expression in BET-inhibitor-resistant NMC cells. MiR-21 regulated the growth of NMCs. The miR-21 inhibitor suppressed the growth of BET-inhibitor-resistant cells. Thirty-two clinical samples were analyzed and NMC was identified using digital PCR assays. Tumors with the *SETD2* mutation were analyzed using NGS.

Conclusions:

We report for the first time SET domain-containing protein 2 (SETD2) deficiency and miR-21 as novel therapeutic targets for NMC. SETD2 loss and miR-21 may be therapeutic targets for NMC.

Trial registration

In this study, we analyzed the gene alterations in human tumor samples. This study was registered in the UMIN clinical trial registered system (UMIN000043147, January 27, 2021).

Background

Nuclear protein in testis (NUT) midline carcinoma (NMC), also referred to as NUT carcinoma, is a rare and highly aggressive tumor with a predilection for the midline structures, affecting both children and adults. NMC is genetically identified by the presence of the *NUT* gene, also known as the NUT midline carcinoma family member 1 (*NUTM1*) gene rearrangement [1, 2]. The most frequent translocation is observed between the *NUT* gene on the 15q14 chromosome and the bromodomain containing 4 (*BRD4*) gene on the 19q13.1 chromosome, which accounts for more than 70% of the rearrangements [3–5]; while the other *NUT* fusion partners include *BRD3*, nuclear receptor binding SET domain protein 3 (NSD3), zinc finger protein 532 (ZNF532), and other genes [6–9]. NMC lacks specific pathological features and can occur in any organ, such as the thorax, head, neck, or other midline organs [10]. Patients with NMC can be misdiagnosed with other malignant tumors due to poor differentiation, unawareness of the disease, and lack of diagnostic tests. The prognosis of patients with NMC is significantly poor, with a median survival time of 6–9 months [3, 10].

The development of novel treatment strategies for NMC is challenging. Pediatric tumors harboring t(15;19) were first reported during 1990s [11, 12]. In 2003, French and colleagues identified the *BRD4-NUT* fusion gene in poorly differentiated midline carcinoma [2]. Studies published from 2000 onwards revealed that the fusion gene plays an important role in NMC development, promoting increased expression of *MYC* and other oncogenes [13]. BRD4 is a member of the bromodomain and extra-terminal domain (BET) family of proteins and binds to acetylated lysine in histones [14]. However, NUT shuttles between the nucleus and the cytoplasm and recruits p300 histone acetyltransferase (HAT) to enhance histone acetylation [6, 8, 15]. Hence, it has been postulated that BRD4-NUT has a predilection for the nucleus where the NUT portion recruits p300 and increases histone acetylation. Then, the fusion protein increases hyperacetylated regions of chromatin up to 2 Mb in size, “megadomains” that drive transcription of the underlying DNA, such as *MYC* and *TP63* [8].

Therefore, targeted therapy studies have been conducted based on the molecular mechanisms underlying aberrant signaling through fusion proteins. BET inhibitors have been investigated, and the first-in-class BET inhibitor JQ1, which competitively binds to bromodomains, has shown prodifferentiative and antiproliferative effects on NMC in preclinical studies [16]. More recently, novel BET inhibitors have been developed, and a BET inhibitor OTX015 (Birabresib) has shown tumor regression and symptomatic relief in two patients with advanced NMC, achieving an overall survival (OS) of 18 months and 19 months; in a phase Ib trial, it showed a 30% response rate (RR) in patients with NMC [17, 18]. In contrast, a phase I/II study demonstrated that another pan-BET inhibitor, GSK525762, showed a 22% RR in patients with NMC [19]. Both inhibitors showed good tolerability in clinical trials; however, the efficacy of BET inhibitors is limited, and NMC can often develop resistance to them [18, 19].

Accordingly, the development of novel treatment strategies, such as combination chemotherapy with BET inhibitors, is required. To date, no other therapeutic targets have been identified in NMC. Studies that focus on simultaneous gene mutations and microRNAs (miRNAs) will help identify novel therapeutic

targets. In this study, we aimed to investigate the role of two epigenetic regulators, SET domain containing 2 (SETD2) and miR-21, as possible therapeutic targets for NMC.

Methods

Cell culture assays and reagents

NMC cell lines Ty82 (RIKEN Cell Bank), HCC2429 (UT Southwestern Medical Center, Dallas, TX, USA), and a lung adenocarcinoma cell line A549 (American Type Culture Collection, USA) were incubated in RPMI 1640 supplemented with 10% FBS and antibiotics at 37°C with 5% CO_2. BET inhibitors JQ-1 and OTX015 and the WEE1 inhibitor, AZD1775 (MK1775, Adavosertib) were purchased from Selleckchem. The BET-inhibitor-resistant HCC2429 cell line was established by exposure to an increasing concentration of JQ-1 (1–100 nM) for 3 months [20].

Cell growth assays were performed using the CellTiter 96 Aqueous One Solution Assay (Promega, USA), and the OxiSelect DNA double-strand break Staining Kit (CELL BIOLABS, USA) was used to detect DNA damage on cells. Immunofluorescence staining was performed according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates (3,000–5,000 cells/well) overnight in triplicates; cells were then treated with the indicated concentrations of drugs for 3 days, and the assays were performed.

Stealth RNAi siRNA (Thermo Fischer Scientific, USA) was used for *NUTM1* knockdown, NUTM1-siRNAs (HSS138007, HSS138008, HSS138009) and Stealth RNAi siRNA Negative Control (#12935300, Thermo Fischer Scientific). Lipofectamine RNAiMAX transfection reagent and Opti-MEM I reduced serum medium (Thermo Fischer Scientific) were used for siRNA transfection. The cells were treated with 50 pmol of siRNA for the indicated times, and the following assays were performed.

miRNA function analysis was performed using miRCURY LNA miRNA mimics and inhibitors (QIAGEN, Japan), hsa-miR-21-5p inhibitor (YI04100689), has-miR-21-5p mimic (YM00473093), miRNA inhibitor controls, and miRNA mimic controls. Lipofectamine 3000 (Thermo Fischer Scientific) and Opti-MEM I reduced serum medium were used for miRNA transfection. In 96-well plates, the cells were treated with 0.66 pmol of miRNA mimics or 5 pmol of miRNA inhibitors for 72 h, and the assays were performed.

Western blotting

NUT (#3625), b-actin (#4967), and horseradish peroxidase (HRP)-labeled anti-rabbit IgG (#7074) antibodies were purchased from Cell Signaling Technology (CST). H3K36me3 (#ab9050) and Histone H3 antibody (#ab1791) were purchased from Abcam. Proteins were extracted from the cells using cell lysis buffer (CST, MA, USA) containing the complete protease inhibitor cocktail (Roche, CA, USA). These antibodies were used at a dilution of 1:1000. NuPAGE gels, Pierce Power Blotter, and iBind Western System (Thermo Fischer Scientific) were used for western blotting. Protein expression was measured using the WSE-6100 LuminoGraph I (ATTO, Korea) and quantified using the CS Analyzer 4 (ATTO).

Next-generation sequencing

Genomic DNA was extracted from the cell lines using the Blood & Cell Culture DNA mini kit (QIAGEN, Japan) and from patient-derived tumors using the QIAamp DNA FFPE Tissue kit (QIAGEN, Japan). DNA was quantified using the Qubit 2.0 fluorometer with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific). We used 50 ng of DNA from cell lines and 10 ng of DNA from tissue samples for PCR amplification using the Ion AmpliSeq Library kit 2.0, and Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fischer Scientific). The Ion Express Barcode Adapters (Thermo Fischer Scientific) were ligated to the PCR products for barcoding the tissue samples. AMPure XP beads (Beckman) were used for PCR product purification. The libraries were sequenced using an Ion PGM System (Thermo Fischer Scientific). DNA sequencing data were obtained using the Torrent Suite ver. 4.0 software (Thermo Fischer Scientific). The variant caller ver. 4.0. is called variants. The reads were aligned with the GRCh38 reference genome. We used the Ion Reporter ver. 5.0, and CLC Genomics Workbench ver. 9.5.1 (QIAGEN) for additional analysis.

Small RNA was extracted from the NMC cell lines using a PureLink miRNA isolation kit (Thermo Fischer Scientific). Small RNA was quantified using the Agilent 2100 Bioanalyzer with an Agilent RNA small RNA kit (Agilent Technologies). A small RNA library was prepared using the Ion Total RNA-Seq Kit v2 (Thermo Fischer Scientific), and the libraries were sequenced on the Ion PGM System. The reads were aligned with miRBase miRNAs. miRNA expression was analyzed using the CLC Genomics Workbench ver. 9.5.1.

MicroRNA assay

Small RNA was extracted from NMC cells using the PureLink miRNA Isolation kit. Small RNA was quantified using a NanoDrop (Thermo Fischer Scientific). miRNA expression was analyzed using TaqMan MicroRNA assays (Thermo Fischer Scientific), mir-21-5p (#000397), and RNU48 (#001006). In this study, RNU48 was used as a housekeeping miRNA. Reverse transcription (RT) was performed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fischer Scientific) and the indicated RT primers in the assay kits. PCR was performed using the QuantStudio 3D Digital PCR Master Mix, QuantStudio 3D Digital PCR 20 K Chip Kit v2, and ProFlex 2x Flat PCR System (Thermo Fisher Scientific). PCR was performed according to the manufacturer's instructions [21]. Absolute quantification was performed using the QuantStudio 3D Digital PCR System (Thermo Fischer Scientific). We analyzed the data using the QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fischer Scientific).

Detection of the BRD4-NUT fusion gene in clinical samples

The Medical Ethics Committee of the Asahikawa Medical University approved the study protocol. Clinical samples from patients with thoracic tumor were collected at the Asahikawa Medical University Hospital between 2015 and 2018, and written informed consent was obtained from all patients. The specific primers and probes for *BRD4-NUT* detection were designed: BRD4-NUT-F, TGAAGGGCTTCTCGTCCTCAG (5'–3'); BRD4-NUT-R, GCGGCACTAGGTTTCATGCTC (5'–3'); and BRD4-NUT FAM probe, TCGGAGAGCTCAGTGAGTCCAGCT (5'–3'). RNeasy Mini Kit (QIAGEN) was used to extract RNA from the

cell lines and the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fischer Scientific) was used to extract RNA from the clinical samples. First-strand cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Thermo Fischer Scientific), and the RT reaction was performed according to the manufacturer's instructions. The QuantStudio 3D Digital PCR Master Mix, QuantStudio 3D Digital PCR 20 K Chip Kit v2, and ProFlex 2x Flat PCR System were used for PCR amplification according to the manufacturer's instructions. Absolute quantification was performed using the QuantStudio 3D Digital PCR System, and QuantStudio 3D Analysis Suite Cloud Software was used for data analysis.

Statistical analysis

The measurements are presented as mean ± SE. Statistical analysis was performed using the GraphPad Prism Software 7.0 (GraphPad Software, USA), and the results were analyzed using Student *t*-test. Two-sided p values <0.05 were considered to be statistically significant.

Results

A SETD2 loss-of-function mutation was detected in NMC

To date, concomitant mutations other than fusion genes have not been fully elucidated in NMC. Additionally, the fusion protein regulates cell growth and epigenesis of NMC, and the role of epigenetic regulators, such as miRNA, remains to be studied in NMC. Therefore, we investigated gene mutations and miRNA expression in NMC using NGS to identify novel therapeutic targets for NMC.

First, we performed gene mutational analysis using the NMC cell lines HCC2429 and Ty82. Using a comprehensive cancer panel for NGS, 10 non-synonymous mutations were identified in HCC2429 cells, while 19 were identified in Ty82 cells (Fig. 1a). Among these mutations, we focused on the novel *SETD2* mutation, *SETD2-p.Ser2382fs*, because SETD2 is an epigenetic regulator. Further, this mutation was common in both cell lines.

SETD2 is a tumor suppressor gene [22-24]. SETD2 is a non-redundant trimethyltransferase responsible for the trimethylation of lysine 36 on histone H3 (H3K36me3). We observed that the frameshift mutation was located on exon 17 before the WW and Set2 Rpb1 interacting (SRI) domains of the *SETD2* gene (Fig. 1b). Hence, it was speculated that the mutation would cause the loss of SETD2 function, i.e., decreased H3K36me3 expression in NMC. Indeed, western blot analysis revealed decreased levels of H3K36me3 in NMC cells, particularly in HCC2429 cells (Fig. 1c).

There is synthetic lethality between H3K36me3 deficiency and WEE1 inhibition: H3K36me3-deficient cancers are significantly sensitive to WEE1 inhibitors, which induce DNA damage in tumors [25]. We then evaluated the anti-tumor efficacy of the WEE1 inhibitor, AZD1775 (MK1775, Adavosertib) in NMC cells. The cell viability assay showed that NMC cells were sensitive to AZD1775 compared with A549 as a control lung cancer cell line harboring wild-type *SETD2* (Fig. 2a). Additionally, the expression levels of γH2AX were increased in NMC cells treated with AZD1775 (Fig. 2b). Furthermore, we investigated the

growth suppression effects of the combination of AZD1775 and JQ-1 (Fig. 2c). As a result, additive effects of the combination were observed, suggesting that the efficacy of WEE1 inhibition is independent of BET inhibition. NMC often acquires resistance to BET inhibitors. We then established HCC2429 cells resistant to BET inhibitors (HCC2429-JQR) by sustained treatment with JQ-1, the HCC2429-JQR cells were resistant to both JQ-1 and another BET inhibitor, OTX015 (MK8628, Birabresib) (Fig. 3a). As expected, the cell growth assay showed that even HCC2429-JQR cells were sensitive to AZD1775 (Fig. 3b).

miR-21 regulated the growth of NMC

Next, we performed miRNA expression analyses. miRNAs are key regulators of epigenetics and pathogenesis in many cancers [26]. However, the relationship between miRNAs and *BRD4-NUT* has not been fully elucidated. To determine changes in miRNA expression by BRD4 or NUT inhibition, we performed RNA-seq on HCC2429 cells treated with *NUT* siRNA or JQ-1. Using WB, HCC2429 cells treated with *NUT* siRNA showed decreased NUT expression (Fig. 4a). The MTS assay showed that *NUT* siRNA had negligible effects on the growth of HCC2429 cells (Fig. 4a). However, RNA-seq analysis revealed that the expression levels of miRNA were quite different between the siRNA-treated and non-treated cells; the most common miRNA was let-7a-1/let-7a-2/let-7a-3 in untreated cells, whereas miR-21-5p (hereafter miR-21) was the most abundant one in siRNA-treated cells (Fig. 4b). In contrast, JQ-1 did not significantly change miR-21 expression in HC2429 cells (Fig. 4b). miR-21 is an oncomiR, and we speculated that miR-21 could be associated with NMC growth [27]. The cell growth effects of miR-21 mimics or inhibitors on NMC were studied. As expected, miR-21 mimics increased the growth of HCC2429 cells; however, miR-21 inhibitors decreased their growth (Fig. 4c).

Short-term treatment with JQ-1 did not alter the expression levels of miR-21 in NMCs (Fig. 4b). However, we suspected that miR-21 is related to BET inhibitor resistance because studies have shown that tumors with acquired resistance to BET inhibitors do not have gatekeeper mutations and drug pump activation [28]. Next, we investigated miR-21 expression in HCC2429-JQR, and the miR-21 expression levels between HCC2429 and HCC2429-JQR were compared using the TaqMan miRNA assay. We found that miR-21 expression was increased in the HCC2429-JQR cells compared to that in the parent cells (Fig. 5a). The MTS assay demonstrated that the miR-21 inhibitor suppressed the growth of HCC2429-JQR cells (Fig. 5b).

Since AZD1775 caused cell growth suppression in the HCC2429-JQR cells as described previously, we determined the efficacy of the combination with the miR-21 inhibitor and AZD1775. Notably, the combination of AZD1775 and the miR-21 inhibitor had additive effects on HCC2429-JQR cells (Fig. 5c).

The SETD2 loss-of-function mutation was detected in a patient with NMC

Finally, we investigated gene mutations in clinical NMC samples. Because the thorax is the most frequent location of NMC, we analyzed the expression of the *NUT* fusion genes in patients with malignant thoracic tumors, whose tumors were located in the midline of their bodies [29]. Thirty-two tumor samples collected via transbronchial biopsy were retrospectively analyzed. Screening for NMC was performed using

immunohistochemistry (IHC), which detected NUT, expressed in the nuclei of NMC cells [30, 31]. However, in some cases, IHC analysis could not be performed because of the lack of tissue, especially in small biopsy samples. In this study, most of the biopsy samples were very small, and it was assumed that these samples included a small number of cancer cells. We then established a highly sensitive dPCR assay to detect the *BRD4-NUT* fusion gene. In our preclinical study, the dPCR assay successfully detected the *BRD4-NUT* fusion gene in 2429 cells; the dPCR assay detected a 1000-fold diluted fusion gene (Fig. 6a). As a result, we identified one NMC tumor among the 32 tumors using the assay (Fig. 6c). Because the recurrent *SETD2* mutation was observed in the NMC cell lines, gene mutational analysis was performed to detect the *SETD2* mutation in the NMC tumor, using the cancer comprehensive panel for NGS. As expected, we identified the *SETD2-p.Ser2382fs* in the tumor.

Discussion

In this study, we describe two epigenetic regulators, SETD2 and miR-21, as therapeutic targets for NMC, regardless of resistance to BET inhibitors. To the best of our knowledge, this is the first report showing the efficacy of targeted therapy with a WEE1 and miR-21 inhibitor in NMC.

First, we identified a novel *SETD2* mutation in NMC. SETD2 has three conserved domains, AWS-SET-PostSET, WW, and Set2 Rpb1 interacting (SRI) domains. The SET domain (1550–1673), which mediates trimethylation of H3K36; the WW domain (2391–2420) is associated with protein-protein interactions, preferentially binding to the proline-rich region of proteins, and the SRI domain (2469–2548) binds to the phosphorylated C-terminal domain of RNA polymerase II, allowing SETD2 to move to transcription elongation complexes [32–34]. Taken together, SETD2 plays a key role in homologous recombination repair and genome stability by catalyzing trimethylation at H3K36 [35].

SETD2 is ubiquitously expressed in human tissues, and somatic mutations of the *SETD2* gene have been reported in several types of cancer, in which loss of SETD2 function leads to decreased H3K36me3 levels [22]. For instance, in clear cell renal cell carcinoma, widespread DNA hypomethylation associated with SETD2 loss-of-function mutations was observed, and the *SETD2* mutation seemed to be related to genomic alterations leading to tumorigenesis [36, 37]. We detected the *SETD2-p.Ser2382fs* mutation in NMC. This frameshift mutation is located just before the WW domain, suggesting that the WW and SRI domains in the mutant SETD2 have no additional normal functions. It has been reported that SRI domain deficiency abolishes trimethylation of H3K36me3 [38, 39]. Indeed, H3K36me3 expression was decreased in NMC cells. On the other hand, H3K36me3 expression levels in Ty82 cells were different from those in HCC2429 cells. Our NGS analysis showed that the frequency of the frameshift mutation was 93.9% in HCC2429 cells and 89% in Ty82 cells; the residual *SETD2* gene was mutant *SETD2*-p.Pro2381Leu (P2381L) in both cell lines (data not shown). Considering the mutation site, the *SETD2-P2381L* mutation may be a passenger mutation. The mutant SETD2-P2381L could compensate for SETD2 deficiency caused by the frameshift mutation in Ty82 cells, although the bona fide activity of the SETD2-P2381L enzyme remains unknown. If there is a monoallelic deficiency of *SETD2* in Ty82 cells, they may retain the trimethyltransferase activity. The mechanistic details remain to be elucidated as to how SETD2-

p.Ser2382fs and SETD2-p.Ser2382fs functions in NMC. In addition, there might be possible mechanisms that compensate for H3K36me3 in Ty82 cells.

Identification of the *SETD2* mutation can lead to the development of targeted therapies for NMC. In H3K36me3-deficient tumors, WEE1 inhibition has a synthetic lethal interaction with H3K36me3 loss; the WEE1 inhibitor AZD1775 selectively kills SETD2-deficient cancer cells through dNTP starvation because of RRM2 depletion [25]. In our study, the NMC cells were more sensitive to AZD1775 than the BET inhibitors, and AZD1775 induced DNA damage in NMC, which was concordant with the results of the present study.

The enzymes regulating epigenesis in histones are categorized as writers, erasers, readers, or others [40, 41]. Among these enzymes, SETD2 belongs to the writers that add post-translational modifications, whereas BRD4 is one of the readers that recognize acetyl groups on histone lysines. Therefore, our findings suggest that aberrant gene expression and tumorigenesis in NMC might occur through hyperacetylation by BRD4-NUT and hypomethylation by SETD2 loss. We found that the combination of the BET inhibitor and WEE1 inhibitor had additive effects on NMC. Moreover, a recent report showed that the combination therapy targeting BET and p300, which belongs to writers and acetylates histone lysine residues, was more effective than BET inhibitor alone [42]. Given the epigenetic categories in histone modification, the combination of BET inhibitors and target inhibitors in another epigenetic category might be useful for the treatment of NMC.

Next, we found that miR-21 regulated the growth of NMC. Emerging studies have reported the functions of miRNAs in NMC. A study showed a set of 48 dysregulated miRNAs in NMC, in which the miRNAs targeting critical genes other than BRD4 and NUT were analyzed; however, miR-21 was not included in the 48 miRNAs [43]. Another study screened an miRNA mimic library and identified miR-3140 that targets and suppresses BRD4 by binding to its coding sequence [44]. Another report analyzed miRNA expression in NMC using clinical samples that identified three cases of sinonasal NMC, and two out of three NMCs showed upregulation of miR-21, miR-143, and miR-484 [45]. miRNA expression is regulated by DNA methylation and histone modifications [46]. Therefore, histone modification changes caused by SETD2 deficiency or BRD4-NUT might be associated with miR-21 expression. Indeed, altered promoter methylation of miR-21 has been reported in SETD2-deficient cancers [36]. Additionally, H3K36me3 is required for DNA mismatch repair (MMR), while miR-21 downregulates MMR gene expression [47, 48]. Overall, together with SETD2 deficiency, miR-21 might be a key regulator in NMC.

We established HCC2429-JQR cells resistant to BET inhibitors. In our study, miR-21 expression was increased in the resistant NMC, and both the miR-21 inhibitor and AZD1775 were effective in the resistant cells. BET family proteins include BRD2, BRD3, BRD4, and BRDT. The BET proteins have conserved tandem bromodomains BD1 and BD2, which selectively bind to acetylated lysine residues in histones. BET inhibitors competitively bind to the individual or both bromodomains and inhibit BET activity [16, 17]. Therefore, tumors resistant to BET inhibitors are expected to have gatekeeper mutations at the bromodomains, as seen in epidermal growth factor receptor mutations in lung cancer [49]. However, it is

unlikely that the tumors acquire resistant mutations. In triple-negative breast cancer (TNBC), gatekeeper mutations, new driver gene alterations, and drug pump activation were not observed in BET-resistant TNBC cells [28]. This was true in other malignant tumors such as ovarian cancer, prostate cancer, and leukemia, in which alternative signaling pathways other than BET itself were associated with acquired resistance [50–53]. Therefore, it is possible that NMC might not acquire gatekeeper mutations, although we did not evaluate the mutations in bromodomains in resistant NMC cells. Recent work has shown that adaptive kinome reprogramming is associated with acquired resistance to targeted therapies, and aberrant kinase activation has occurred in BET-resistant cancer cells without gatekeeper mutations [50, 54]. miR-21 potentially targets more than 400 genes, using data from miRDB (http://www.mirdb.org), which include various genes of receptor tyrosine kinases (RTKs). Therefore, an increase in miR-21 might be associated with acquired resistance to BET inhibitors by activating RTKs and downstream pathways in NMC. Therapeutic targets other than BET, NUT, and their associated proteins have not been reported. We demonstrated the efficacy of AZD1775 and a miR-21 inhibitor, which could overcome resistance.

Conclusions

Our comprehensive study found SETD2 deficiency and miR-21 as therapeutic targets for NMC. WEE1 and miR-21 inhibitors are novel therapeutic options for NMC and should be investigated in clinical trials.

List Of Abbreviations

NMC Nut midline carcinoma

NUT Nuclear protein in testis

NUTM1 NUT midline carcinoma family member 1

BRD4 bromodomain containing 4

NSD3 SET domain protein 3

ZNF532 Zinc finger protein 532

BET Bromodomain and extra-terminal domain

HAT Histone acetyltransferase

RR Response rate

miRNA Micro RNA

RT Reverse Transcription

SETD2 SET domain containing 2

HRP horseradish peroxidase

WB Western blotting

NGS Next-generation sequencing

dPCR Digital PCR

IHC Immunohistochemistry

SRI Set2 Rpb1 interacting

MMR Mismatch repair

TNBC Triple-negative breast cancer

RTK Receptor tyrosine kinase

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the Asahikawa Medical University. Written informed consent was obtained from all patients.

Consent for publication

Written informed consent was obtained from all patients.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NY performed the gH2AX assay. SO performed the growth assays, NGS experiments, data analysis, knockdown experiments, miRNA assays, and dPCR assays, and was a major contributor in writing the manuscript. TS collected biopsy samples from the patients. SC performed NGS and analyzed the data.

MS performed a historical examination of thoracic tumors. KO performed the gH2AX assay. RY established a BET-inhibitor-resistant NMC cell line. NH, YM, MK, and YO collected biopsy samples. All authors read and approved the final manuscript.

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References

1. French CA, Miyoshi I, Aster JC, Kubonishi I, Kroll TG, Dal Cin P, Vargas SO, Perez-Atayde AR, Fletcher JA. BRD4 bromodomain gene rearrangement in aggressive carcinoma with translocation t(15;19). Am J Pathol. 2001;159(6):1987–92.
2. French CA, Miyoshi I, Kubonishi I, Grier HE, Perez-Atayde AR, Fletcher JA. BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma. Cancer Res. 2003;63(2):304–7.
3. French CA. NUT Carcinoma: Clinicopathologic features, pathogenesis, and treatment. Pathol Int. 2018;68(11):583–95.
4. Bauer DE, Mitchell CM, Strait KM, Lathan CS, Stelow EB, Lüer SC, Muhammed S, Evans AG, Sholl LM, Rosai J, et al. Clinicopathologic features and long-term outcomes of NUT midline carcinoma. Clin Cancer Res. 2012;18(20):5773–9.
5. Chau NG, Hurwitz S, Mitchell CM, Aserlind A, Grunfeld N, Kaplan L, Hsi P, Bauer DE, Lathan CS, Rodriguez-Galindo C, et al. Intensive treatment and survival outcomes in NUT midline carcinoma of the head and neck. Cancer. 2016;122(23):3632–40.
6. French CA, Ramirez CL, Kolmakova J, Hickman TT, Cameron MJ, Thyne ME, Kutok JL, Toretsky JA, Tadavarthy AK, Kees UR, et al. BRD-NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells. Oncogene. 2008;27(15):2237–42.
7. French CA, Rahman S, Walsh EM, Kühnle S, Grayson AR, Lemieux ME, Grunfeld N, Rubin BP, Antonescu CR, Zhang S, et al. NSD3-NUT fusion oncoprotein in NUT midline carcinoma: implications for a novel oncogenic mechanism. Cancer Discov. 2014;4(8):928–41.
8. Alekseyenko AA, Walsh EM, Zee BM, Pakozdi T, Hsi P, Lemieux ME, Dal Cin P, Ince TA, Kharchenko PV, Kuroda MI, et al. Ectopic protein interactions within BRD4-chromatin complexes drive oncogenic megadomain formation in NUT midline carcinoma. Proc Natl Acad Sci U S A. 2017;114(21):E4184–92.
9. Shiota H, Elya JE, Alekseyenko AA, Chou PM, Gorman SA, Barbash O, Becht K, Danga K, Kuroda MI, Nardi V, et al. "Z4" Complex Member Fusions in NUT Carcinoma: Implications for a Novel Oncogenic Mechanism. Mol Cancer Res. 2018;16(12):1826–33.
10. French CA. Pathogenesis of NUT midline carcinoma. Annu Rev Pathol. 2012;7:247–65.

11. Kubonishi I, Takehara N, Iwata J, Sonobe H, Ohtsuki Y, Abe T, Miyoshi I: **Novel t(15;19)(q15;p13) chromosome abnormality in a thymic carcinoma**. *Cancer Res* 1991, **51**(12):3327–3328.
12. Kees UR, Mulcahy MT, Willoughby ML. Intrathoracic carcinoma in an 11-year-old girl showing a translocation t(15;19). Am J Pediatr Hematol Oncol. 1991;13(4):459–64.
13. Grayson AR, Walsh EM, Cameron MJ, Godec J, Ashworth T, Ambrose JM, Aserlind AB, Wang H, Evan G, Kluk MJ, et al. MYC, a downstream target of BRD-NUT, is necessary and sufficient for the blockade of differentiation in NUT midline carcinoma. Oncogene. 2014;33(13):1736–42.
14. Dhalluin C, Carlson JE, Zeng L, He C, Aggarwal AK, Zhou MM. Structure and ligand of a histone acetyltransferase bromodomain. Nature. 1999;399(6735):491–6.
15. Reynoird N, Schwartz BE, Delvecchio M, Sadoul K, Meyers D, Mukherjee C, Caron C, Kimura H, Rousseaux S, Cole PA, et al. Oncogenesis by sequestration of CBP/p300 in transcriptionally inactive hyperacetylated chromatin domains. EMBO J. 2010;29(17):2943–52.
16. Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM, Keates T, Hickman TT, Felletar I, et al. Selective inhibition of BET bromodomains. Nature. 2010;468(7327):1067–73.
17. Noel JK, Iwata K, Ooike S, Sugahara K, Nakamura H, Daibata M. Abstract C244: Development of the BET bromodomain inhibitor OTX015. Mol Cancer Ther. 2013;12(11 Supplement):C244–4.
18. Lewin J, Soria JC, Stathis A, Delord JP, Peters S, Awada A, Aftimos PG, Bekradda M, Rezai K, Zeng Z, et al. Phase Ib Trial With Birabresib, a Small-Molecule Inhibitor of Bromodomain and Extraterminal Proteins, in Patients With Selected Advanced Solid Tumors. J Clin Oncol. 2018;36(30):3007–14.
19. Piha-Paul SA, Hann CL, French CA, Cousin S, Braña I, Cassier PA, Moreno V, de Bono JS, Harward SD, Ferron-Brady G, et al. Phase 1 Study of Molibresib (GSK525762), a Bromodomain and Extra-Terminal Domain Protein Inhibitor, in NUT Carcinoma and Other Solid Tumors. JNCI Cancer Spectr. 2020;4(2):pkz093.
20. Tsuji T, Ozasa H, Aoki W, Aburaya S, Yamamoto Funazo T, Furugaki K, Yoshimura Y, Yamazoe M, Ajimizu H, Yasuda Y, et al. YAP1 mediates survival of ALK-rearranged lung cancer cells treated with alectinib via pro-apoptotic protein regulation. Nat Commun. 2020;11(1):74.
21. Conte D, Verri C, Borzi C, Suatoni P, Pastorino U, Sozzi G, Fortunato O. Novel method to detect microRNAs using chip-based QuantStudio 3D digital PCR. BMC Genom. 2015;16:849.
22. Li J, Duns G, Westers H, Sijmons R, van den Berg A, Kok K. SETD2: an epigenetic modifier with tumor suppressor functionality. Oncotarget. 2016;7(31):50719–34.
23. Fahey CC, Davis IJ. **SETting the Stage for Cancer Development: SETD2 and the Consequences of Lost Methylation**. *Cold Spring Harb Perspect Med* 2017, 7(5).
24. McDaniel SL, Strahl BD. Shaping the cellular landscape with Set2/SETD2 methylation. Cell Mol Life Sci. 2017;74(18):3317–34.
25. Pfister SX, Markkanen E, Jiang Y, Sarkar S, Woodcock M, Orlando G, Mavrommati I, Pai CC, Zalmas LP, Drobnitzky N, et al. Inhibiting WEE1 Selectively Kills Histone H3K36me3-Deficient Cancers by dNTP Starvation. Cancer Cell. 2015;28(5):557–68.

26. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov. 2017;16(3):203–22.
27. Bautista-Sánchez D, Arriaga-Canon C, Pedroza-Torres A, De La Rosa-Velázquez IA, González-Barrios R, Contreras-Espinosa L, Montiel-Manríquez R, Castro-Hernández C, Fragoso-Ontiveros V, Álvarez-Gómez RM, et al. The Promising Role of miR-21 as a Cancer Biomarker and Its Importance in RNA-Based Therapeutics. Mol Ther Nucleic Acids. 2020;20:409–20.
28. Shu S, Lin CY, He HH, Witwicki RM, Tabassum DP, Roberts JM, Janiszewska M, Huh SJ, Liang Y, Ryan J, et al. Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. Nature. 2016;529(7586):413–7.
29. French CA. The importance of diagnosing NUT midline carcinoma. Head Neck Pathol. 2013;7(1):11–6.
30. Haack H, Johnson LA, Fry CJ, Crosby K, Polakiewicz RD, Stelow EB, Hong SM, Schwartz BE, Cameron MJ, Rubin MA, et al. Diagnosis of NUT midline carcinoma using a NUT-specific monoclonal antibody. Am J Surg Pathol. 2009;33(7):984–91.
31. Park HS, Bae YS, Yoon SO, Lim BJ, Hong HJ, Ro JY, Hong SW. Usefulness of Nuclear Protein in Testis (NUT) Immunohistochemistry in the Cytodiagnosis of NUT Midline Carcinoma: A Brief Case Report. Korean J Pathol. 2014;48(4):335–8.
32. Gao YG, Yang H, Zhao J, Jiang YJ, Hu HY. Autoinhibitory structure of the WW domain of HYPB/SETD2 regulates its interaction with the proline-rich region of huntingtin. Structure. 2014;22(3):378–86.
33. Li M, Phatnani HP, Guan Z, Sage H, Greenleaf AL, Zhou P. Solution structure of the Set2-Rpb1 interacting domain of human Set2 and its interaction with the hyperphosphorylated C-terminal domain of Rpb1. Proc Natl Acad Sci U S A. 2005;102(49):17636–41.
34. Edmunds JW, Mahadevan LC, Clayton AL. Dynamic histone H3 methylation during gene induction: HYPB/Setd2 mediates all H3K36 trimethylation. EMBO J. 2008;27(2):406–20.
35. Pfister SX, Ahrabi S, Zalmas LP, Sarkar S, Aymard F, Bachrati CZ, Helleday T, Legube G, La Thangue NB, Porter AC, et al. SETD2-dependent histone H3K36 trimethylation is required for homologous recombination repair and genome stability. Cell Rep. 2014;7(6):2006–18.
36. Network CGAR. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature. 2013;499(7456):43–9.
37. Chiang YC, Park IY, Terzo EA, Tripathi DN, Mason FM, Fahey CC, Karki M, Shuster CB, Sohn BH, Chowdhury P, et al. Haploinsufficiency for Microtubule Methylation Is an Early Driver of Genomic Instability in Renal Cell Carcinoma. Cancer Res. 2018;78(12):3135–46.
38. Kizer KO, Phatnani HP, Shibata Y, Hall H, Greenleaf AL, Strahl BD. A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol Cell Biol. 2005;25(8):3305–16.
39. Li B, Howe L, Anderson S, Yates JR, Workman JL. The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. J Biol Chem.

2003;278(11):8897–903.
40. Tarakhovsky A. Tools and landscapes of epigenetics. Nat Immunol. 2010;11(7):565–8.
41. Bates SE. Epigenetic Therapies for Cancer. N Engl J Med. 2020;383(7):650–63.
42. Morrison-Smith CD, Knox TM, Filic I, Soroko KM, Eschle BK, Wilkens MK, Gokhale PC, Giles F, Griffin A, Brown B, et al. Combined Targeting of the BRD4-NUT-p300 Axis in NUT Midline Carcinoma by Dual Selective Bromodomain Inhibitor, NEO2734. Mol Cancer Ther. 2020;19(7):1406–14.
43. Pathak E, Bhavya, Mishra D, Atri N, Mishra R. Deciphering the Role of microRNAs in BRD4-NUT Fusion Gene Induced NUT Midline Carcinoma. Bioinformation. 2017;13(6):209–13.
44. Tonouchi E, Gen Y, Muramatsu T, Hiramoto H, Tanimoto K, Inoue J, Inazawa J. miR-3140 suppresses tumor cell growth by targeting BRD4 via its coding sequence and downregulates the BRD4-NUT fusion oncoprotein. Sci Rep. 2018;8(1):4482.
45. Laco J, Kovarikova H, Chmelarova M, Vosmikova H, Sieglova K, Bubancova I, Dundr P, Nemejcova K, Michalek J, Celakovsky P, et al. Analysis of DNA methylation and microRNA expression in NUT (nuclear protein in testis) midline carcinoma of the sinonasal tract: a clinicopathological, immunohistochemical and molecular genetic study. Neoplasma. 2018;65(1):113–23.
46. Handa H, Murakami Y, Ishihara R, Kimura-Masuda K, Masuda Y. **The Role and Function of microRNA in the Pathogenesis of Multiple Myeloma**. *Cancers (Basel)* 2019, 11(11).
47. Yamamoto H, Imai K. Microsatellite instability: an update. Arch Toxicol. 2015;89(6):899–921.
48. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, Easton J, Chen X, Wang J, Rusch M, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012;481(7380):157–63.
49. Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med. 2005;352(8):786–92.
50. Kurimchak AM, Shelton C, Duncan KE, Johnson KJ, Brown J, O'Brien S, Gabbasov R, Fink LS, Li Y, Lounsbury N, et al. Resistance to BET Bromodomain Inhibitors Is Mediated by Kinome Reprogramming in Ovarian Cancer. Cell Rep. 2016;16(5):1273–86.
51. Jin X, Yan Y, Wang D, Ding D, Ma T, Ye Z, Jimenez R, Wang L, Wu H, Huang H. DUB3 Promotes BET Inhibitor Resistance and Cancer Progression by Deubiquitinating BRD4. Mol Cell. 2018;71(4):592–605.e594.
52. Pawar A, Gollavilli PN, Wang S, Asangani IA. Resistance to BET Inhibitor Leads to Alternative Therapeutic Vulnerabilities in Castration-Resistant Prostate Cancer. Cell Rep. 2018;22(9):2236–45.
53. Rathert P, Roth M, Neumann T, Muerdter F, Roe JS, Muhar M, Deswal S, Cerny-Reiterer S, Peter B, Jude J, et al. Transcriptional plasticity promotes primary and acquired resistance to BET inhibition. Nature. 2015;525(7570):543–7.
54. Duncan JS, Whittle MC, Nakamura K, Abell AN, Midland AA, Zawistowski JS, Johnson NL, Granger DA, Jordan NV, Darr DB, et al. Dynamic reprogramming of the kinome in response to targeted MEK

inhibition in triple-negative breast cancer. Cell. 2012;149(2):307–21.

Figures

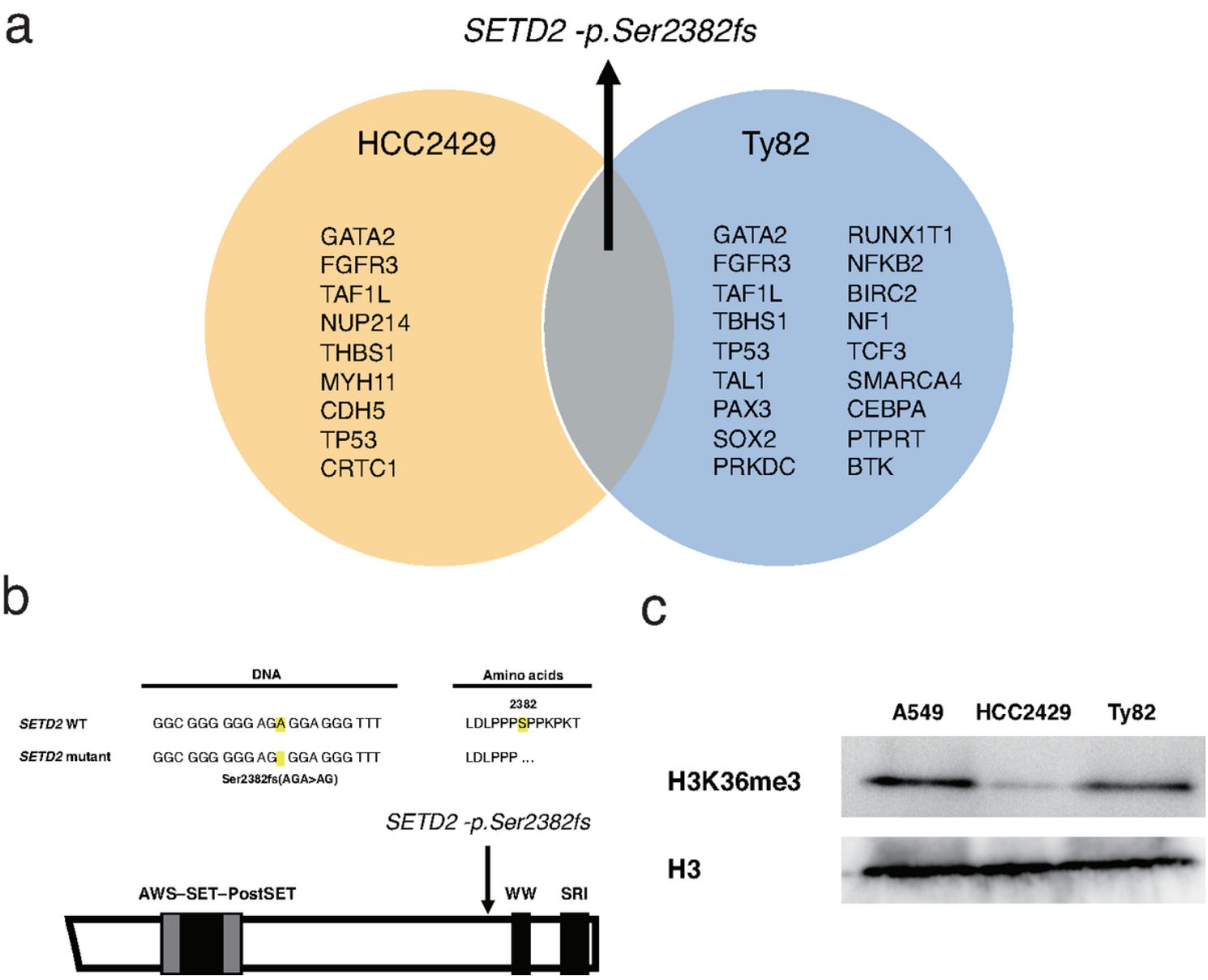

Figure 1

NMC with the recurrent SETD2 mutation. a. A list of concurrent gene mutations in the NMC cell lines HCC2429 and Ty82. Next-generation sequencing identified the recurrent SETD2-p.Ser2382fs mutation. b. The SETD2 frameshift mutation in the HCC2429 cells. The mutation is located before the WW domain. c. H3K36me3 expressions in WB. The H3K36me3 expression is decreased in NMC compared with A549, a lung adenocarcinoma cell line.

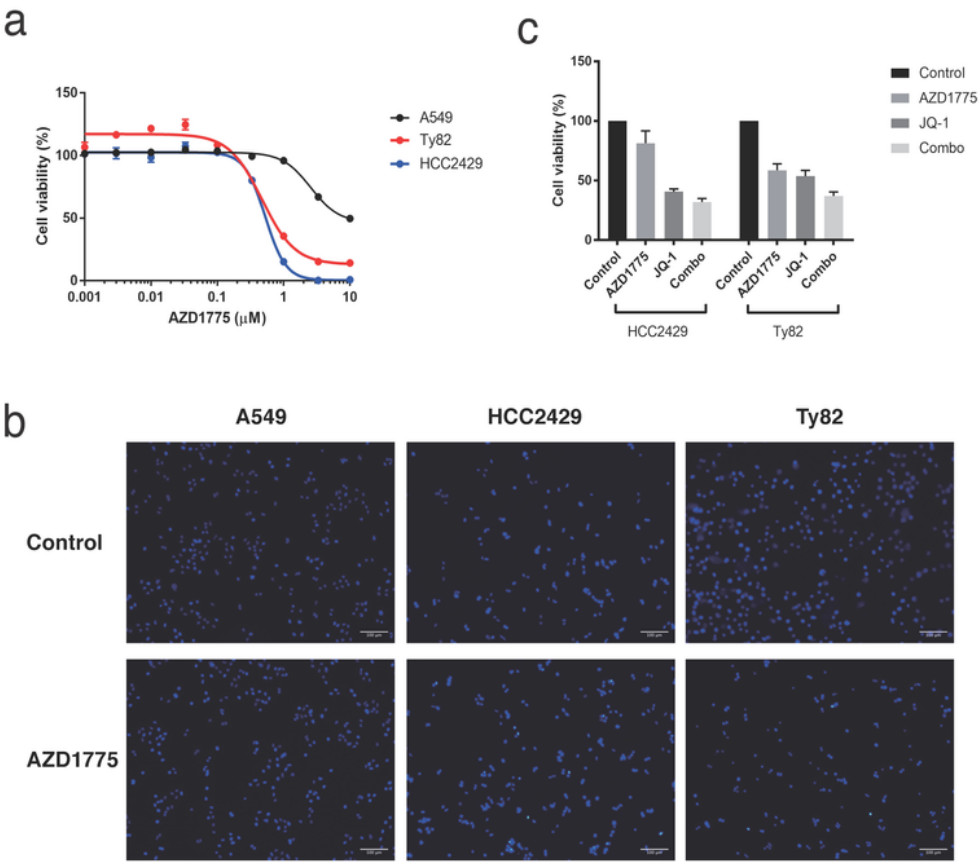

Figure 2

The NMC cells are sensitive to the WEE1 inhibitor, AZD1775. a. MTS assays comparing sensitivity to AZD1775 between NMCs and A549. The cells were treated with the indicated concentrations of AZD1775 for 3 days. The NMC cells were sensitive to AZD1775 compared with A549. b. γH2AX assays in cells. Treatment with AZD1775 increased γH2AX expressions (FITC, green) in HCC2429 and Ty82 cells. c. MTS

assays studying the efficacy of the combination with AZD1775 and a BET inhibitor JQ-1. NMC cells were treated with 0.3 μM of AZD1775, 30 nM of JQ-1, or the combination for 3 days.

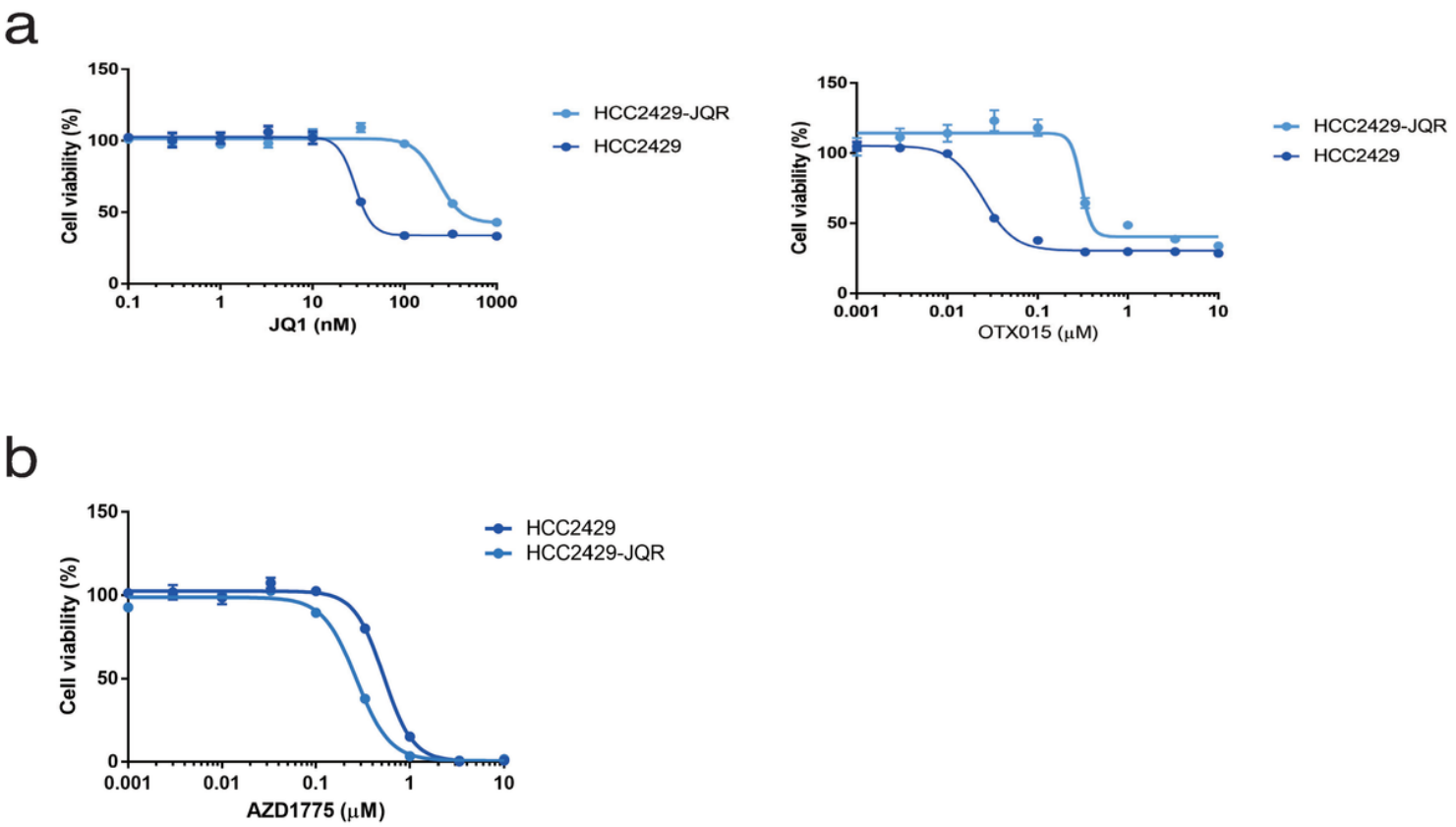

Figure 3

The BET-inhibitor-resistant HCC2429 cells (HCC2429-JQR) are sensitive to AZD1775. a. MTS assays comparing sensitivity to BET inhibitors in the parent HCC2429 and HCC2429-JQR. The cells are treated with BET inhibitors JQ-1 and OTX015 for 3 days. HCC2429-JQR is resistant to BET inhibitors. b. MTS assays in HCC2429 and HCC2429-JQR. These cells were treated with AZD1775 for 3 days. HCC2429-JQR cells were sensitive to AZD1775.

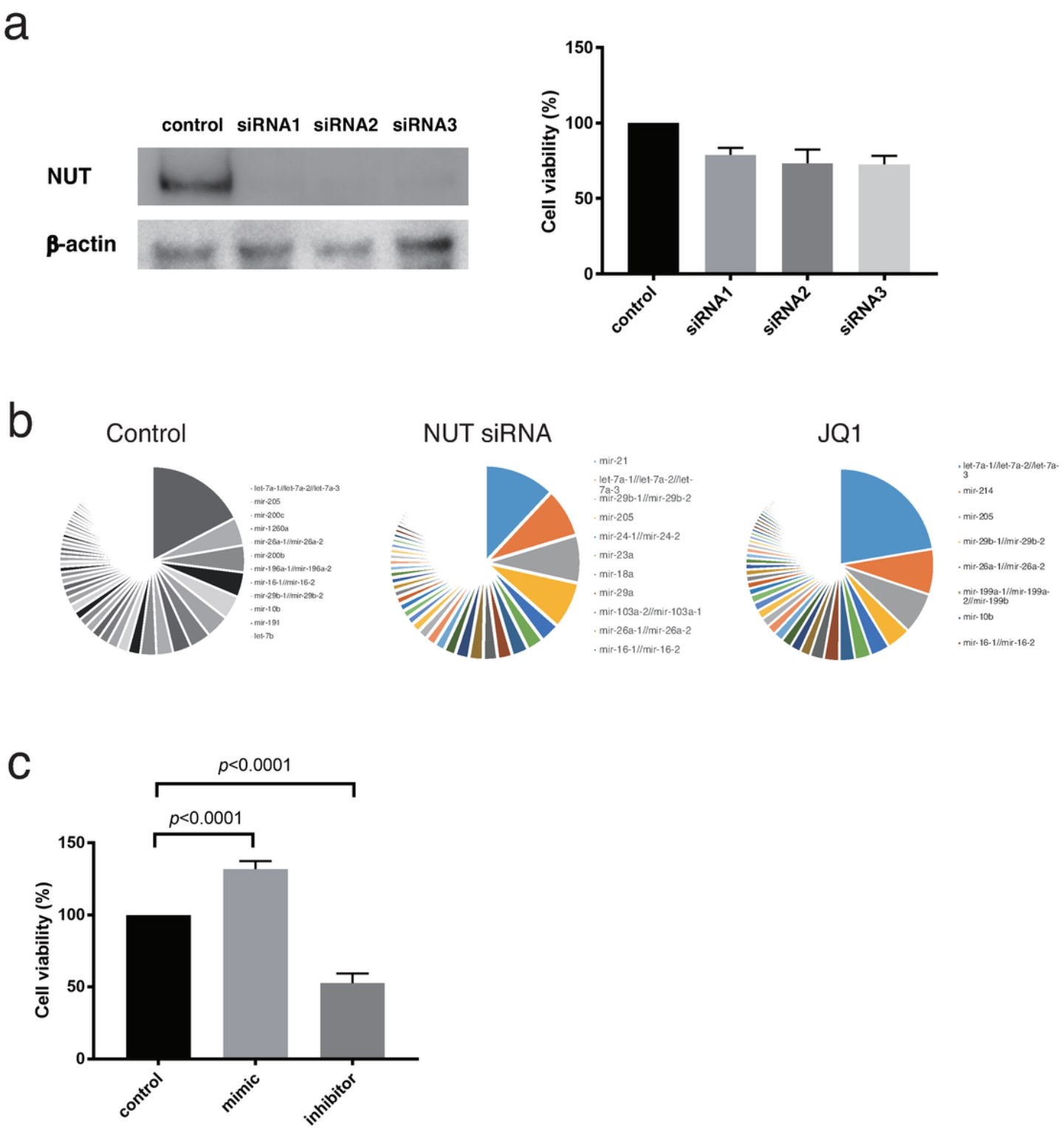

Figure 4

An altered miR-21 expression is observed in HCC2429, and miR-21 regulates HCC2429 cell growth. a. The efficacy of NUT knockdown in HCC2429. HCC2429 cells were treated with NUT siRNAs for 3 days. WB demonstrated decreased NUT expression via NUT knockdown. MTS assay showed partial suppression of cell growth using NUT siRNAs. b. MiRNA expression profiles in HCC2429. RNA sequencing was performed in HCC2429 treated with NUT siRNA1–3 or JQ-1. Compared with control, miR-21 was increased in the

NUT-siRNA-treated cells. c. MTS assays studying the miR-21 mimic or miR-21 inhibitor in HCC2429. The miR-21 mimic and inhibitor regulated the growth of HCC2429.

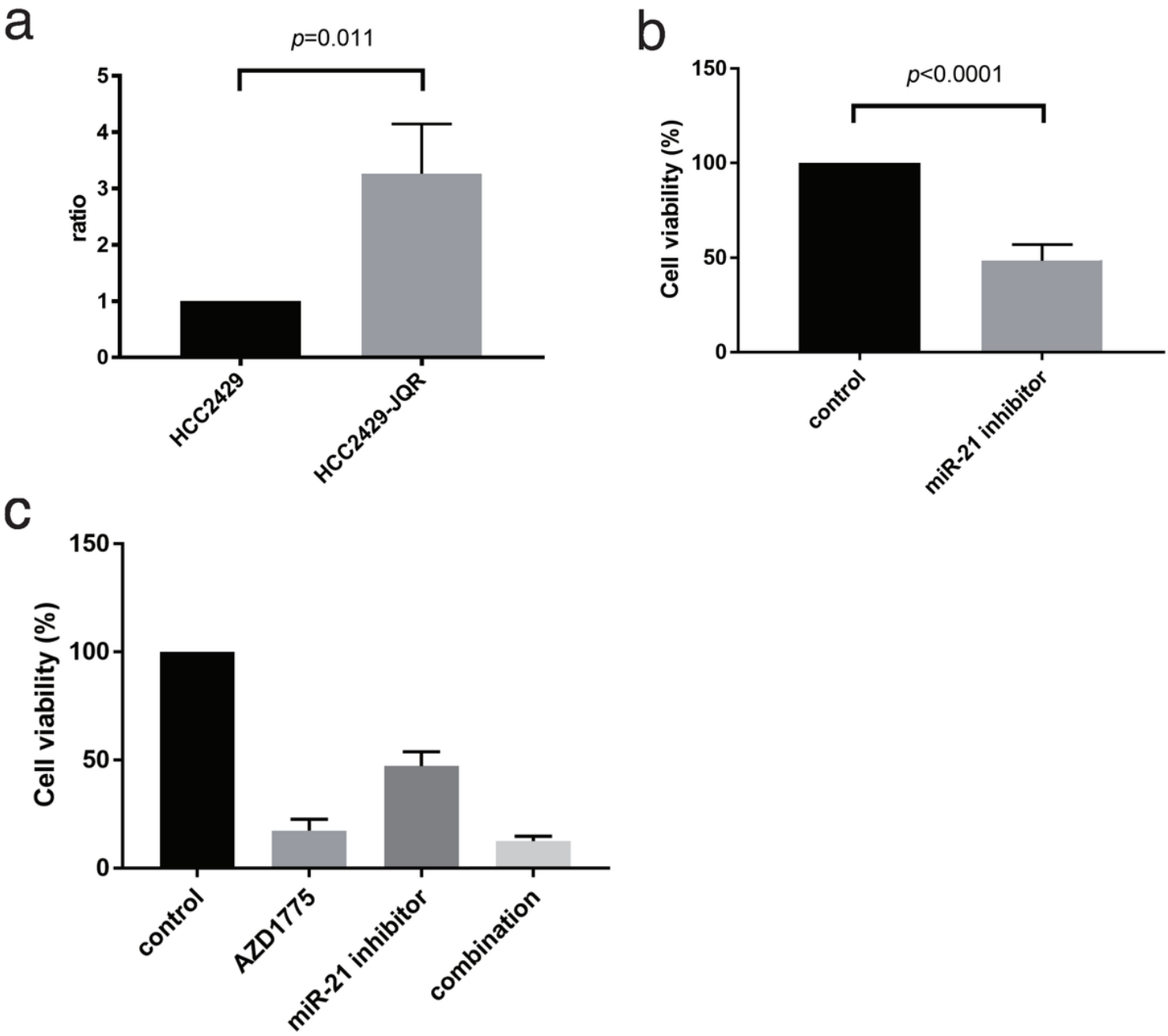

Figure 5

MiR-21 expression was increased in HCC2429-JQR. a. MiRNA-based assays in HCC2429 and HCC2429-JQR. MiR-21 expression was significantly increased in HCC2429-JQR compared with HCC2429. b. MTS assays studying the efficacy of the miR-21 inhibitor in HCC2429-JQR. HCC2429-JQR cells were treated with the miR-21 inhibitor for 3 days. The miR-21 inhibitor suppressed the growth of HCC2429-JQR. c. MTS assays in HCC2429-JQR treated with a combination of AZD1775 and miR-21 inhibitors. The HCC2429-JQR cells were treated with 1.0 M of AZD1775, the miR-21 inhibitor, or the combination for 3 days.

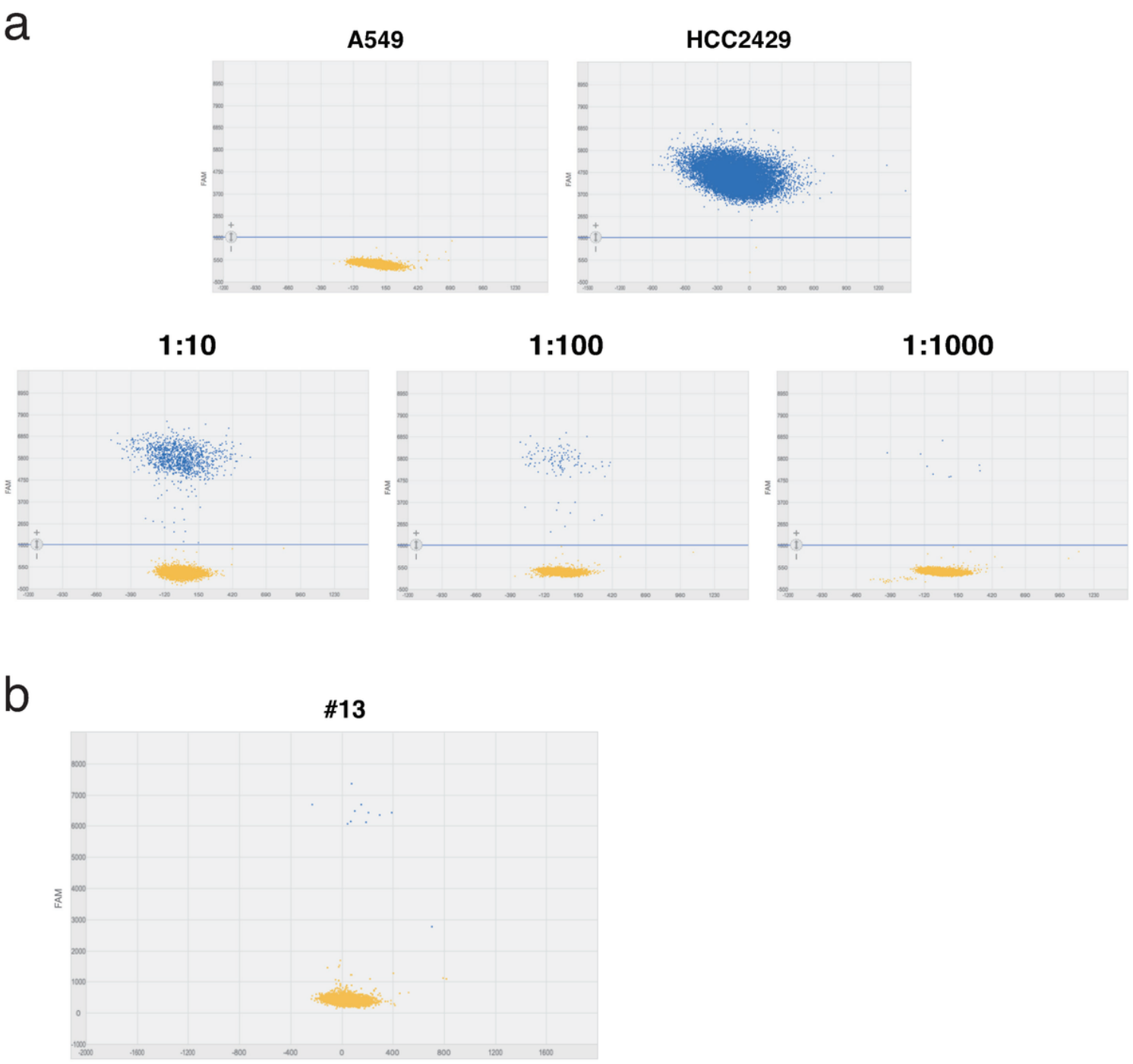

Figure 6

Digital PCR (dPCR) assays identified BRD4-NUT in NMC. a. The dPCR assays in HCC2429 and A549. We designed the specific probe for BRD4-NUT (FAM). The probe successfully identified BRD4-NUT in HCC2429, at up to a 1000-fold dilution. b. The dPCR assays in clinical samples. The assay identified the BRD4-NUT in #13 patients' tumor.