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Ribonucleotide reductase represents a novel therapeutic target in primary effusion lymphoma

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Abstract

Primary effusion lymphoma (PEL) is a highly aggressive B-cell malignancy that is closely associated with one of oncogenic viruses infection, Kaposi's sarcoma-associated herpesvirus (KSHV). PEL prognosis is poor and patients barely survive more than 6 months even following active chemotherapy interventions. There is therefore an urgent need to discover more effective targets for PEL management. We recently found that the ribonucleotide reductase (RR) subunit M2 is potentially regulated by the key oncogenic HGF/c-MET pathway in PEL (Dai *et al.*, *Blood.* 2015;126(26):2821-31). In the current study, we set to investigate the role of RR in PEL pathogenesis and to evaluate its potential as a therapeutic target. We report that the RR inhibitor 3-AP actively induces PEL cell cycle arrest through inhibiting the activity of the NF-κB pathway. Using a xenograft model, we found that 3-AP effectively suppresses PEL progression in immunodeficient mice. Transcriptome analysis of 3-AP treated PEL cell lines reveals altered cellular genes, most of whose roles in PEL have not yet been reported. Taken together, we propose that RR and its signaling pathway may serve as novel actionable targets for PEL management.

Keywords

KSHV; RRM2; primary effusion lymphoma; PEL; 3-AP

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Introduction

HIV+ individuals have a greater risk of developing malignancies including lymphomas, even with successful antiretroviral treatment. The oncogenic Kaposi's sarcoma–associated herpesvirus (KSHV) can cause several human cancers including primary effusion lymphoma (PEL), that is often seen in the HIV/AIDS population.[1] PEL comprises transformed B cells which usually harbors the KSHV episome and preferentially arises within the peritoneal or pleural cavities of immunocompromised patients.[2] PEL is a highly progressive malignancy, since a median survival time is only about 6 months even after the conventional chemotherapy.[3-5] The poor prognosis is mainly due to the evolving multidrug resistance of PEL cells[6] and it is still largely unclear how drug resistance develops. Therapeutic failure is also caused by interactions between the cART (combined AntiRetroviral Treatment) agents and chemotherapeutic drugs. For instance, nonnucleoside reverse-transcriptase inhibitors (NNRTIs) and protease inhibitors can alter CYP3A4 pharmacokinetics, which has been found involved in the metabolism of many chemotherapeutic agents.[7] Thus, it still requires to discover new targets for improving PEL treatment.

We have recently shown that the signaling pathway of Hepatocyte Growth Factor (HGF)/c-MET is highly activated in KSHV+ PEL cells. [8] One of the selective c-MET inhibitors, PF-2341066, can significantly induce apoptosis in PEL cells by causing cell cycle arrest as well as DNA damage, and it can effectively suppress tumor progression in a xenograft model. [8] Our transcriptome analysis shows that the ribonucleotide reductase subunit M2 (RRM2) is regulated by the HGF/c-MET signaling in PEL cells. Ribonucleotide reductase (RR) is a potential target for developing anticancer agents because of its unique role in DNA synthesis, tumor growth, metastasis, and chemoresistance.[9] The human RR is composed of two subunits, α subunits (RRM1) and β subunits (RRM2). The α subunits contain one catalytic site and 2 binding sites for those varied enzyme regulators. The β subunits (RRM2) can recruit a binuclear iron cofactor and generate a stable tyrosyl radical for the catalysis reactions.[10] Normal cells generally express low levels of RR, whereas neoplastic cells usually express high levels of RR to produce dNTPs required for the DNA synthesis as well as cell proliferation.[10-12]

We recently reported that caspase-dependent apoptosis of KSHV+ PEL cells were induced by either RNAi-mediated silencing of RRM2 or by the RRM2 inhibitor, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP).[8] However, how RR regulates PEL survival and its therapeutic target potential within PEL remain largely unknown. Here, we found that 3-AP actively induces PEL cell cycle arrest through the inhibition of NF-κB signaling pathway. Using a xenograft model, we found that the 3-AP can effectively suppress PEL progression *in vivo*. Further, unique transcriptome signatures were identified in 3-AP treated PEL cell lines.

Results and discussion

3-AP mediated inhibition of RRM2 prevents KSHV+ PEL cell proliferation through a G1 cell cycle arrest

Using the WST-1 cell proliferation assays (Roche),[13,14] we found that 3-AP treatment dramatically suppressed the proliferation of all four examined KSHV+ PEL cell lines (BCBL-1, BCP-1, BC-1 and BC-3) in a dose-dependent manner (Figs. 1a-1d). The 50% Inhibitory Concentration (IC_{50}) of 3-AP ranged from 0.4 to 0.7 μM. Consistently, RNAi mediated silencing of RRM2 showed a similar reduction of PEL proliferation, indicating a key role of RRM2 in PEL proliferation (Fig. S1). Next, our flow cytometry analysis showed that the 3-AP induced a G1 cell cycle arrest of PEL (Fig. 1e). Subsequent immunoblots analysis indicated that 3-AP regulated the expression of several key cell cycle check-point factors including the down-regulation of Cyclin D1, CDK6, and phosphor-Rb and up-regulation of p16 and p21 within BC-1 and BCBL-1 cells (Figs. 1f-1g). At the transcriptional level, we found that 3-AP significantly reduced CDK6 RNA levels while increasing p21 transcripts (Fig. S2), although the underlying regulatory mechanisms still remain to be investigated, such as through p53-dependent or independent mechanisms.[15] In fact, p53 gene mutations seldom occur in PEL cells, although p53 is usually inactive in PEL due to the relative stoichiometries of varied complexes between LANA, hdm2, and p53.[16,17] Interestingly, p21 has been found as a direct target by one of the KSHV microRNAs, miR-K1, which finally attenuating p21-mediated cell cycle arrest in PEL.[18] Consistent with 3-AP treatment, a G1 cell cycle arrest was observed by RNAi mediated silencing of RRM2 in PEL (Fig. S3).

We also tested the impact of 3-AP on viral gene expression profiles in PEL cells, but we found that 3-AP only slightly reduced the expression of latent genes (e.g. *LANA*) and increased the expression of lytic genes (e.g. *RTA, K8.1, vGPCR, ORF57*) within BC-1 and BCBL-1 cells (Fig. S4), probably due to its rapid “killing” influence on tumor cells.

Suppression of the NF-κB pathway is required for the 3-AP induced PEL apoptosis and cell cycle arrest

We next examined several signaling pathways commonly involved in PEL proliferation and survival.[19] Our results indicated that 3-AP significantly inhibited the phosphorylation of NF-κB p65, but showed no effects on Akt and MAPK-ERK phosphorylation in PEL cells (Figs. 2a-2b). Interestingly, we found that 3-AP also negatively regulated the RRM2 expression. To further confirm whether the NF-κB signaling pathway is affected by 3-AP, we performed a NF-κB reporter assay[20] and found that 3-AP significantly suppressed the activity of NF-κB in both BCBL-1 and BC-1 cells (Fig. 2c). Next, we transfected a NF-κB p65 plasmid[20] into PEL cells and found that the enforced expression of NF-κB successfully attenuated 3-AP induced apoptosis and cell cycle arrest (Figs. 2d-2f). Taken together, our results support the notion that the NF-κB pathway is required for 3-AP induced PEL apoptosis and cell cycle arrest.

3-AP suppresses PEL tumor progression in a xenograft murine model

Next, we examined whether 3-AP can suppress PEL tumor growth in an established xenograft murine model.[21] All the protocols have been approved by the Louisiana State University Health Science Center Animal Care and Use Committee, which is also in accordance with national guidelines. BCBL-1 cells were first intraperitoneally (i.p.) injected into the NOD/SCID mice. Twenty-four hours later, 3-AP (or vehicle) was given by i.p. injection, once daily for 3 days per week and the dose (20 mg/kg) was maintained over a period of 5-weeks. Our results showed that 3-AP dramatically suppressed PEL progression, manifested by decreased ascites formation and spleen enlargement (Figs. 3a-3c). A high level of tumor infiltration was found in the spleens of vehicle-treated mice but not in mice treated with 3-AP (Fig. 3d). Meanwhile, western blot analysis showed a dramatic down-regulation of phosphor-p65 and CDK6 as well as an up-regulation of p21 expression in spleen tissues of 3-AP treated mice (Fig. 3e). Thus, our results indicated that 3-AP affects PEL survival through the similar mechanisms both *in vitro* and *in vivo*.

Transcriptome analysis of 3-AP treated PEL cell lines

We used Illumina microarray to investigate the transcriptome change between 3-AP and vehicle treated PEL cell lines (BCP-1, BC-1 and BCBL-1). The intersection analysis showed that a total of 34 genes were significantly altered among all three 3-AP treated cell lines (expression change ≥2 fold and $p<0.05$); 34 genes were altered in BCP-1, BC-1 and BCBL-1; 65 genes were altered in both BC-1 and BCBL-1 cells; 48 genes were altered in both BCP-1 and BCBL-1 cells; 45 genes were altered in both BC-1 and BCP-1 cells; 30 genes were altered only in BCBL-1 cells; 21 genes were altered only in BCP-1 cells; 512 genes were altered only in BC-1 cells (Fig. 4a). We reasoned that the co-infection of the Epstein-Barr virus (EBV) and KSHV may contribute to the much higher number of gene alterations observed in BC-1 cells. The heat map of commonly altered 34 genes was shown in Fig. 4b, and more detailed information on gene expression changes can be found in Table S1.

Among the 34 commonly altered genes, some small nuclear RNAs such as *RN7SK*, *RNVU1-18*, *RNU1-4, RNU1-1* and *RNU6-1* were highly up-regulated and such alterations have also been observed with the treatment of KSHV+ PEL cell lines with the c-MET inhibitor (PF-2341066).[8] Genes related to tumor cell proliferation, such as *AURKA* (Aurora kinase A) was significantly down-regulated in 3-AP treated PEL cell lines. The Aurora kinase family, and in particular Aurora A, is required for multiple mitotic events and its aberrant expression is related to tumorigenesis.[22] To date, a number of inhibitors targeting Aurora A, B and pan-Aurora kinase have been approved by the FDA for cancer management, such as for ovarian cancer and acute myelogenous leukemia (AML).[23] Another commonly down-regulated gene is *HMMR* (Hyaluronan-mediated motility receptor), also known as the receptor for hyaluronan-mediated motility (RHAMM). *HMMR* is an oncogene and it plays critical roles in the neoplastic progression of leukemia and several solid tumors.[24] Interestingly, our previous study has reported that the hyaluronan signaling is involved in PEL multidrug chemoresistance.[6] Since the roles of most of these altered genes in PEL pathogenesis are still unknown, we will continue exploring their functions in future studies. To assess the specificity of these commonly altered genes in 3-

AP treated KSHV+ PEL cell lines, we selected 10 of them (5 up-regulated and 5 down-regulated, respectively) and compared their transcriptional change between vehicle and 3-AP treated BL-41 (a KSHV negative lymphoma cell line[19]) cells using qRT-PCR. This analysis indicated that some genes are also significantly altered in 3-AP treated BL-41 cells (e.g. *LY96*, *RN7SK*, *SNORD3C*, *KIF20A*), while the others show no significant changes (e.g. *CCL3L3*, *PPP1R15A*, *FAM72A*, *HMMR*), implying the specificity of the latter in 3-AP treated PEL cell lines (Fig. S5).

We next performed enrichment analysis of all three gene sets (Set I: genes that were commonly changed in all the three cell lines. Set II: genes that were changed in two cell lines. Set III: genes that were changed in only one cell line) using Pathway map, Gene Ontology (GO) Processes, and Process Networks modules of the Metacore Software (Thompson Reuters).[8] This analysis showed that several major cellular functions were affected within 3-AP treated PEL cell lines, including the regulation of cell cycle, apoptosis/programmed cell death, cytoskeleton/spindle microtubules, and progesterone signaling (Figs. 4c-4e). The top 2 identified pathway maps and protein networks are listed in Figs. S6 and S7, respectively.

Our results first time demonstrate that ribonucleotide reductase (RR) might serve as a novel therapeutic target for KSHV+ PEL. The RR inhibitor 3-AP can effectively suppress PEL progression in a xenograft immunodeficient murine model. Although the application of current FDA approved RR inhibitors (e.g. hydroxyurea, 3-AP, and GTI2040) is limited by their short half-life, iron chelation and drug resistance,[25-27] improved RR inhibitors might soon prove their values in cancer management. For instance, Zhou *et al* have recently identified a novel and promising RR inhibitor, COH29,[9] that can bind to its ligand-binding pocket and result in blocking the RRM1-RRM2 quaternary structure assembly. Excitingly, COH29 can effectively inhibit the proliferation of majority of the NCI 60 human cancer cell lines, but show little effect on normal fibroblasts and endothelial cells.[9] Currently, we are in the process of testing the efficacy of COH29 in our PEL xenograft model.

3-AP has been shown to be involved in the iron chelation by inhibiting the alternate β subunit, p53R2.[28] Interestingly, cellular iron content might also play a role in the tumorigenesis,[29] since iron can activate RR in tumor cells.[30] Epidemiological studies indicate that iron may contribute to Kaposi Sarcoma (KS) development, another KSHV associated cancer.[31] Thus, the iron withdrawal strategy may be a reasonable choice for the KS management. Simonart *et al* have shown that the chemically unrelated iron chelators such as deferiprone and desferrioxamine (DFO) can inhibit KS growth and induce KS cell apoptosis.[32] Thus, it's likely that 3-AP might also suppress KS growth through iron chelation. We are currently testing the effects of 3-AP in our recently established KS-like nude mouse model using KSHV long-term-infected telomerase immortalized human umbilical vein endothelial cells (TIVE-LTC).[33] Finally, we will expand the study of RR-targeted therapy to other virus associated malignancies such as EBV+ lymphomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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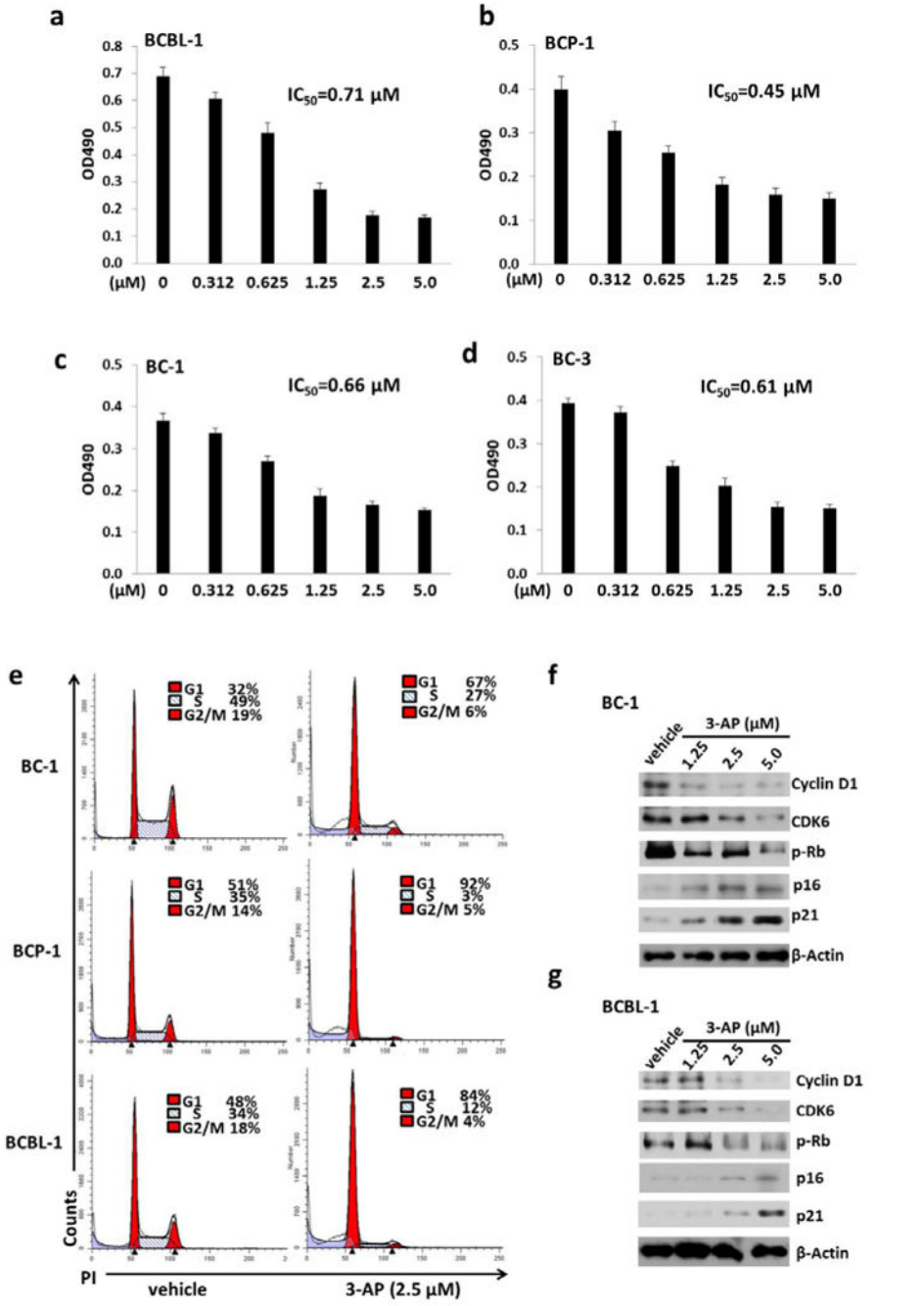

Figure 1. 3-AP prevents KSHV+ PEL proliferation through G1 cell cycle arrest

(**a-d**) A total of four KSHV+ PEL cell lines (BCBL-1, BCP-1, BC-1 and BC-3 purchased from American Type Culture Collection) were incubated with the indicated concentrations of 3-AP for 48 h. The cell proliferation status was examined using the WST-1 cell proliferation assays (Roche). Error bars represent the S.D. for 3 independent experiments. The 50% Inhibitory Concentration (IC_{50}) was calculated by using SPSS 20.0. (**e-g**) PEL cells were incubated with the indicated concentrations of 3-AP for 24 h, and then stained by propidium iodide (PI) and analyzed by flow cytometry. Protein expression was analyzed by

immunoblot analysis. The antibodies for Cyclin D1, CDK6, p-Rb, p16, p21 and β-Actin were purchased from Cell Signaling (Cat. #2978, #3136, #8516, #4824, #2947, #4970).

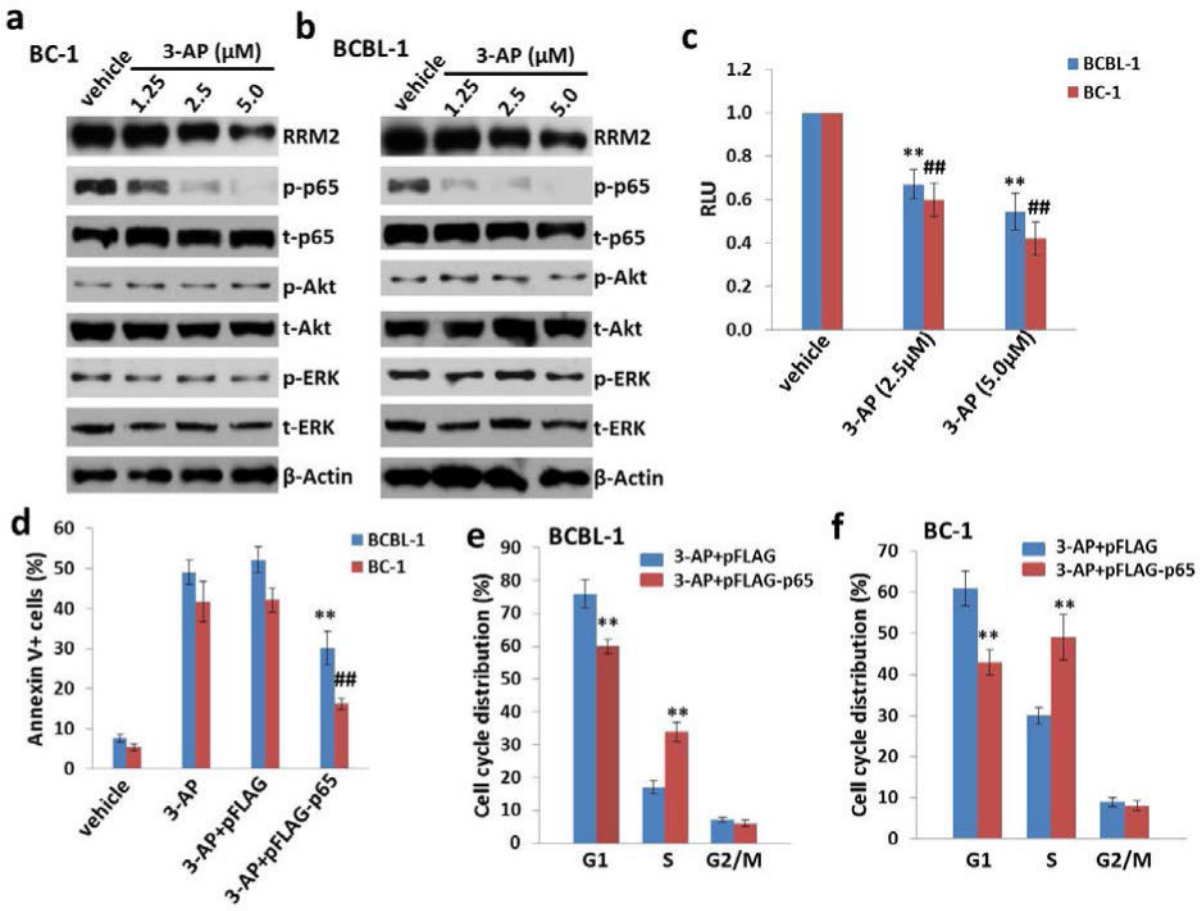

Figure 2. Suppression of the NF-κB pathway is required for 3-AP induced PEL apoptosis and cell cycle arrest

(**a-b**) PEL cells were incubated with the indicated concentrations of 3-AP for 24 h, and protein expression was analyzed by immunoblot analysis. The antibodies for p-ERK/t-ERK, p-Akt/t-Akt, p-p65/t-p65, and β-Actin were purchased from Cell Signaling (Cat. #4370, #4695, #4060, #9272, #3033, #8242, #4970), and RRM2 from Santa Cruz (Cat. #sc-398294). (**c**) PEL cells were first transfected with an NF-κB luciferase reporter construct. Twenty-four hours later, cells were incubated with the indicated concentrations of 3-AP for additional 24 h. Cells were harvested and lysed with 100 μL of lysis buffer (Promega), and 20 μL aliquots from each lysate were quantified for luciferase analysis using a Berthold FB12 luminometer. Light units were normalized to total protein levels for each sample using the BCA protein assay kit (Pierce) to determine the relative luciferase units (RLU). (**d-f**) PEL cells were first transfected with a vector encoding NF-κB p65 (pFLAG-p65) or a control vector (pFLAG). Twenty-four hours later, cells were incubated with 2.5 μM of 3-AP for an additional 24 h. Apoptosis was then quantitatively assessed using the FITC-Annexin V/propidium iodide (PI) Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's instructions. Error bars represent the S.D. for 3 independent experiments. **/## = $p<0.01$ (the 2-tailed Student *t* test, *vs* vehicle or 3-AP+pFLAG group, respectively).

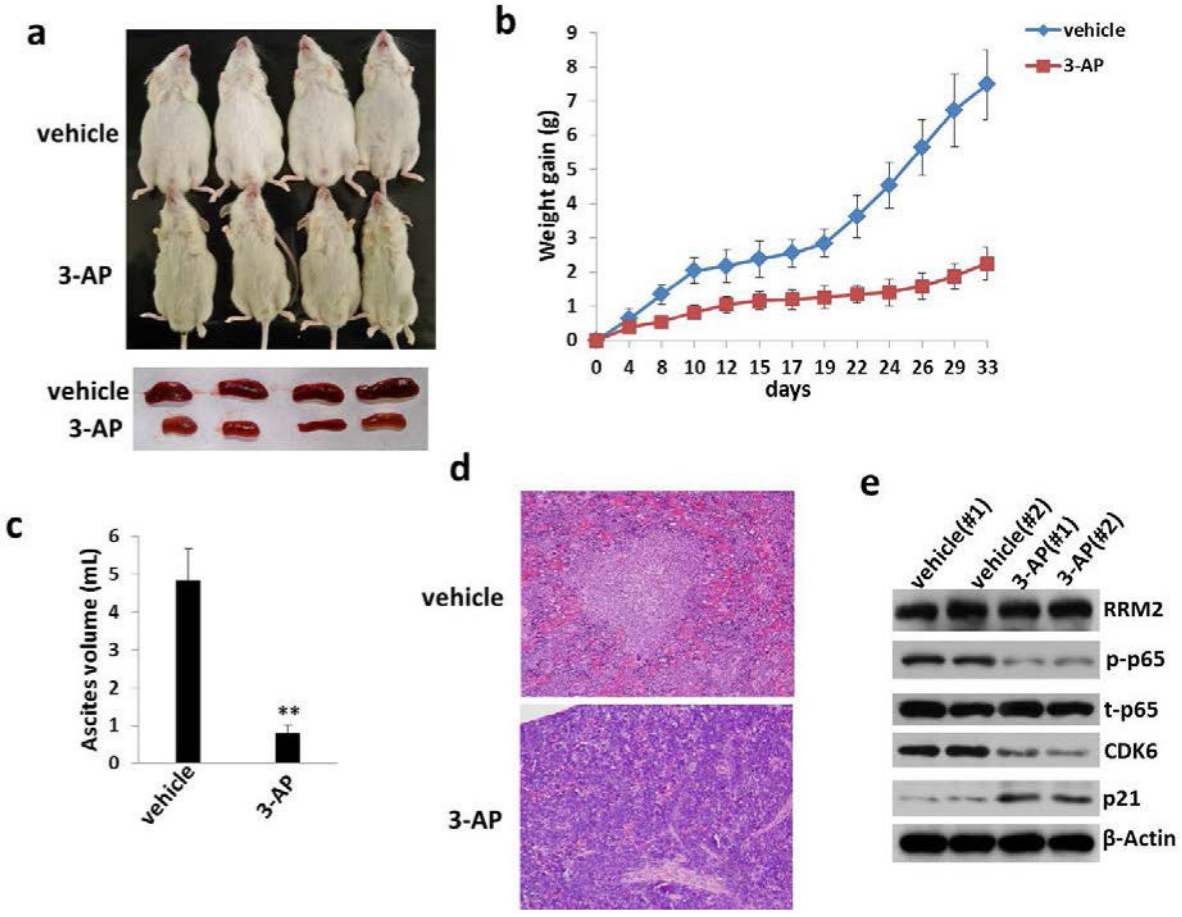

Figure 3. 3-AP treatment suppresses PEL progression *in vivo*

(**a-c**) NOD/SCID mice (6-8 week-old, male) were injected i.p. with 1×10^7 BCBL-1 cells. Twenty-four hours later, 20 mg/kg of 3-AP or vehicle (n=8 per group) were administered i.p., once daily, 3 days per week, for each of 2 independent experiments. Weights were recorded weekly. Images of representative animals and their spleens, as well as ascites fluid volumes, were collected at the conclusion of experiments on day 33. (**d**) Spleens from representative vehicle or 3-AP treated mice were prepared for the routine hematoxylin-and-eosin (H&E) staining. (**e**) Protein expression of the ascites PEL cells collected from the representative vehicle or 3-AP treated mice was analyzed by immunoblot analysis. Error bars represent the S.D. for 1 of 2 independent experiments; ** = $p<0.01$.

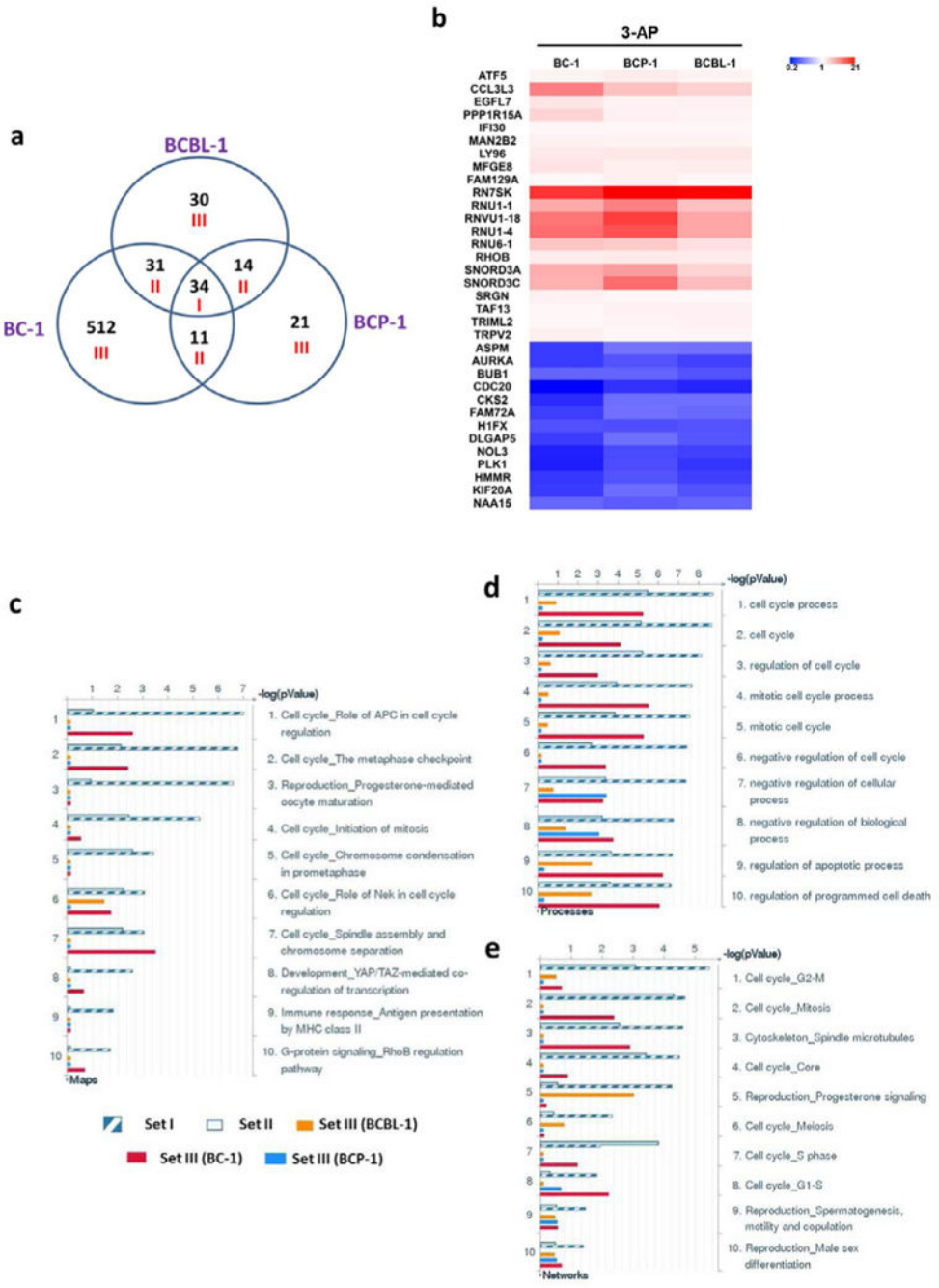

Figure 4. Transcriptome analysis of the 3-AP treated PEL cell lines

(**a**) The Human HT-12 v4 Expression BeadChip (Illumina) was used to investigate the transcriptome change between 3-AP and vehicle treated KSHV+ PEL cell lines (BCBL-1, BC-1 and BCP-1). The BeadChip contains more than 47,000 probes derived from the NCBI RefSeq Release 38 and other sources. The microarray original data have been submitted to Gene Expression Omnibus (GEO) database (Accession number: GSE91389). The intersection analysis of significantly altered genes (expression change ≥ 2-fold and $p<0.05$) was conducted using Illumina GenomeStudio software. Set I: genes that were commonly

altered in all three cell lines. Set II: genes that were altered in two cell lines. Set III: genes that were altered in only one cell line. (**b**) Heat map of genes commonly altered in all three 3-AP treated PEL cell lines (*vs* the vehicle treated controls). The heat map plot was generated by Microsoft Excel 2010. (**c-e**) The enrichment analysis of gene profiles (Set I, II, and III) altered by 3-AP treatment was conducted using the MetaCore software (Thompson Reuters) modules: Pathway Maps, Gene Ontology Processes, and Process Networks.