16 October 2022

Dear Editor,

We appreciate very much for the constructive comments and suggestions. In terms of the comments, we have extensively revised the manuscript entitled “Inhibition of BRD4 enhances the migration of esophageal squamous cell carcinoma cells by inducing cell autophagy” (manuscript ID: 79089). All updated parts have been highlighted in the revised manuscript in yellow. The detailed responses to the comments are to be found below.

Comments

1. Introduction should be shortened.

Reply: We thank the reviewer for this suggestion, and carefully shortened “Introduction” in terms of the context throughout the revised version of our manuscript. We removed references 11 and 12, in which JQ1 inhibited tumor cell proliferation and migration, and have deleted “Multiple clinical phase 1 trials on BET inhibitors for hematological and solid cancers, such as ABBV-075, are ongoing”. Moreover, we have deleted the sentence “Therefore, understanding the molecular mechanisms of metastasis in ESCC will facilitate the discovery of new therapeutic strategies to promote novel drug development with the goal of improving patient survival”. We also changed and deleted some sentences slightly.

2. Results: a reduction of E-Cadherin was found after application of JQ1, an inhibitor of BRD4. In this phenomenon the authors show evidence for epithelial-mesenchymal transition. How do the authors exclude a reorganization of intercellular contacts in a epithelial cell? What about the synthesis of plaque-associated proteins and the expression of keratins?

Reply: We thank the reviewer for the sincere comments. We have supplemented the results that reveal the morphology changes of KESE-450 and KYSE-150 cells after JQ1 treatment in Figure 1B in the revised manuscript. As shown in Figure 1B, JQ1 treatment caused KYSE-450 and KYSE-150 cells from cobblestone morphology which is a typical epithelia cell feature changing to an elongated spindle-like appearance which is the morphological character of mesenchymal cells, indicating that JQ1 results in the esophageal squamous cell carcinoma (ESCC) cells occurring epithelia-mesenchymal transition (EMT). This result is further supported by the western blot results shown as Figure 1D, i.e. JQ1 downregulating E-cadherin (an epithelia cell marker) and upregulating vimentin (a mesenchymal cell marker). One important intercellular contact is adherent junction which major component is E-cadherin. Thus, the reorganization of adherent junction is regulated by the level of E-cadherin. When EMT occurs, the intercellular contacts are weakening. Based on our results and adherent junction features, we consider that JQ1 might weaken intercellular contacts by downregulation of E-cadherin level and this speculation will need verification in our future study by E-cadherin immunofluorescence staining.

We previously had performed the transcriptome RNA sequencing in JQ1-treated and untreated KYSE-450 cells by Personalbio (Shanghai, China; project No. SP18121723). In terms of the comments, we have carefully re-reviewed our RNAseq data which showed that JQ1 reduced the mRNA level of keratin 80 which is the only deferential gene of keratin family proteins analyzed by DEseq analysis (Table 1). Moreover, the KEGG pathway analysis results showed that three genes were up-regulated (Table 2) and seven genes were down-regulated (Table 3) in focal adhesion pathway. Cell migration is a complicated process associated with multiple cellular signaling pathways which are involved in EMT, intercellular contact, organization of cytoskeletal proteins, focal adhesion, *et al*. The comments point out our future plan in order to elucidate comprehensive mechanisms by which JQ1 or inhibition of BRD4 promote cancer cell migration. We will future perform RT-qPCR to verify the results of RNAseq listed in the following tables.

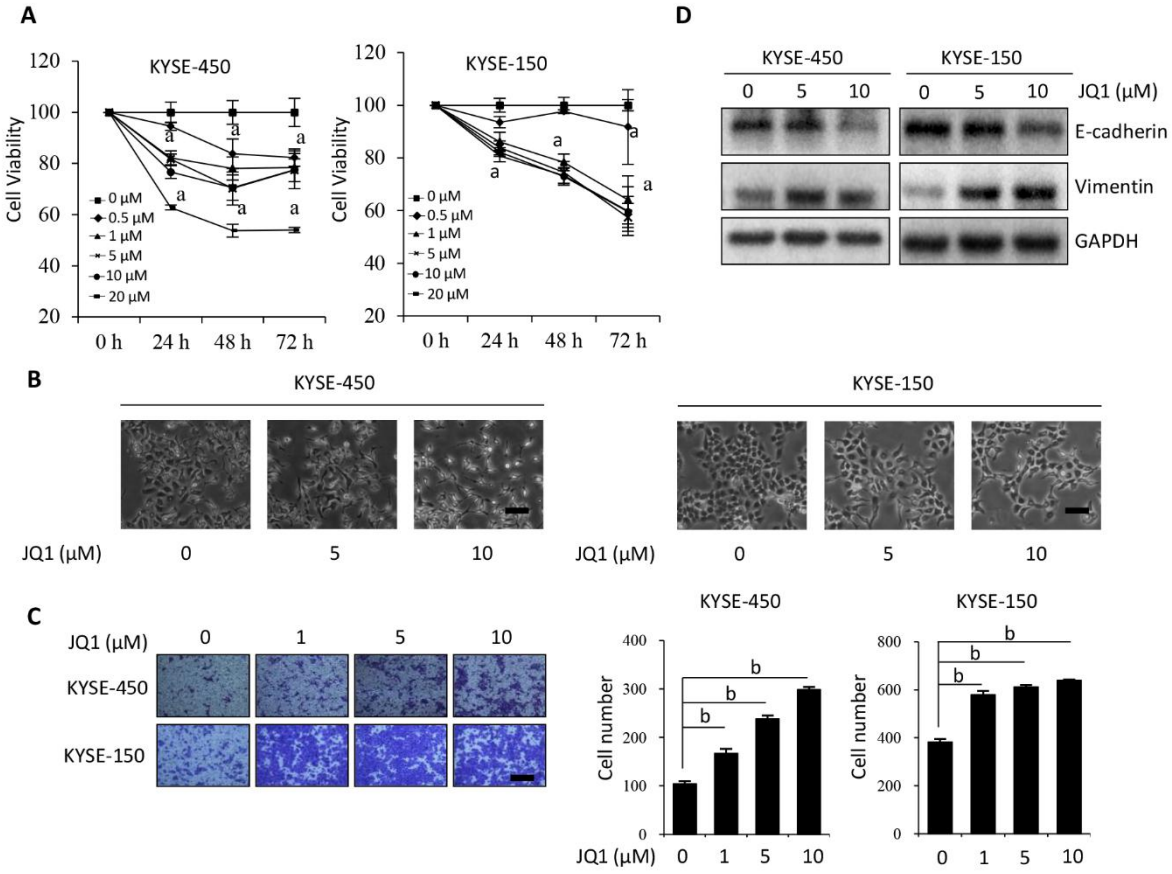

Figure 1 Effects of JQ1, an inhibitor of BRD4, on ESCC cell proliferation and migration. A: MTT assay was conducted to detect cell viability of KYSE-450 cells and KYSE-150 cells after treatment with JQ1 at various fixed dosages at different time points; B: Phase contrast images of ESCC cells treated with JQ1 were captured by a Nikon digital microscope. C: Transwell assay was performed to examine cell migration in KYSE-450 cells and KYSE-150 cells after treatment with JQ1 at various fixed dosages; D: Western blot was carried out to measure the expression levels of E-cadherin and vimentin in KYSE-450 cells and KYSE-150 cells after treatment with JQ1 at various fixed dosages. GAPDH was used as the protein loading control. $^{a}P < 0.05$, $^{b}P < 0.01$. Scale bars in (B) and (C) are 100 μm..

Table 1 DEseq analysis of keratin 80 level in JQ1-treated and untreated KYSE-450 cells

Control	JQ1	Fold change	P value	Name	Description
5805.74788	1182.908067	0.20374775	0.002829828	*KRT80*	keratin 80

Table 2 Upregulation of focal adhesion associated genes in JQ1-treated and untreated KYSE-450 cells

Control	JQ1	Fold change	P value	Name	Description
6381.6937	19091.664	2.991629679	0.0315993	*ITGA3*	integrin subunit alpha 3
161.12241	594.26246	3.688266905	0.0415841	*PIK3R3*	phosphoinositide-3-kinase regulatory subunit 3
63.202714	324.65378	5.136706343	0.0312418	*TLN2*	talin 2

Table 3 Downregulation of focal adhesion associated genes in JQ1-treated and untreated KYSE-450 cells

Control	JQ1	Fold change	P value	Name	Description
8011.6116	2162.4863	0.269919008	0.0119127	*CAV2*	caveolin 2
267.05372	40.441301	0.15143508	0.0227653	*VEGFC*	vascular endothelial growth factor C
37030.559	7230.006	0.195244311	0.0017063	*CAV1*	caveolin 1
698.79057	146.03803	0.20898684	0.0139755	*ITGA4*	integrin subunit alpha 4
1048.6309	128.06412	0.122125064	0.0007604	*TNC*	tenascin C
6810.76	2328.7449	0.341921448	0.0378644	*THBS1*	thrombospondin 1
394.34933	95.486406	0.242136602	0.0452535	*PDGFB*	platelet derived growth factor subunit B

3 Results: autophagy is addressed as important molecular mechanism: it could be of interest the type of molecules/ proteins or organelles that are addressed.

Reply: We appreciate this suggestion very much. In this study，we have discovered that JQ1 facilitates ESCC cell migration and this promoting role is related with JQ1-induced autophagy. Specifically, we found that JQ1 activated AMPK, which activation has been demonstrated inducing autophagy by activating multiple downstream signaling pathways such as activating Beclin-1 and class III PI3K complex signaling, phosphorylating autophagy-related proteins mTORC1 and ULK1. Regarding the comments, we have discussed the potential downstream molecules of AMPK in JQ1-induced autophagy in the manuscript and will explore the related detail mechanisms in future.

4. Results: how do the authors demonstrate that the BRD4 effects are specific for esophageal ESCC? Is there any difference between p16 expressing or non-expressing cells?

Reply: Thank you for this question. Based on the comment, we have observed the effect of JQ1 on human glioma cell line U251 cell migration by transwell assay. As shown in Figure 2, JQ1 treatment caused U251cell morphology to an elongated spindle-like appearance and obviously promoted U251 cell migration in a dose-dependent manner. On the basis of our observations, we speculate that the promoting effect of BRD4 inhibitor on cell migration is not ESCC cell specific. We are planning to determine whether this role occurs on other types of cancer cell so as to provide a reference for developing BRD4 inhibitor combination therapies targeting to activated migration process when clinical application of BRD4 inhibitor as anti-cancer therapy.

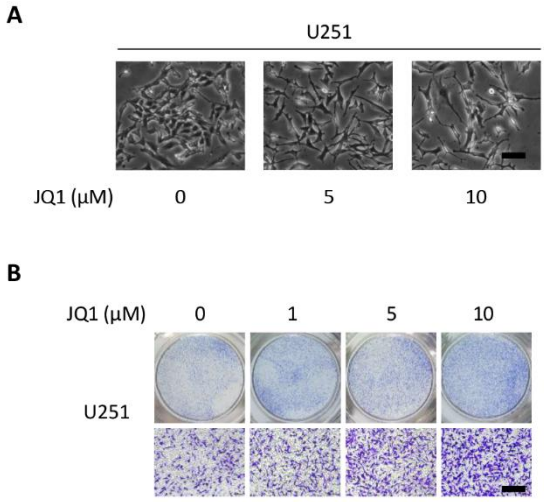

Figure 2 The effect of JQ1 on U251 cell morphology and migration. A. Phase contrast images of U251 cells treated with JQ1 were captured by a Nikon digital microscope. B. Transwell assay was used for detection the ability of cell migration in U251

cells after treated JQ1 with various concentrations. Scale bars in (A) and (B) are 100 μm.

P16-INK4A as an anti-cancer gene has been found homozygous deletion and mutation in 50% of human tumor cell lines. We browsed DepMap portal (https://depmap.org/portal), an online database of cancer cell line encyclopedia (CCLE), and this database reveals that P16-INK4A gene in KYSE150 and KYSE450 cells which were used in our study does not exist mutation, fusion and translocation. Moreover, P16-INK4A methylation has been demonstrated in multiple caner types and is associated with poor prognosis. Such as P16-INK4A methylation was associated with the ESCC development (Fan Z, et al. Feasibility of using P16-INK4A methylation as a cytologic marker for esophageal squamous cell carcinoma screening: A pilot study. Cancer Med. 2022, PMID: 35352503). Thus, the functional state of P16-INK4A is dependent on multiple factors including gene mutation, protein level as well as post-translational modification, *et al*. In terms of the comment, we further examined the protein levels of P16-INK4A in KYSE-450 and KYSE-150 cells as well as glioma cell line U251 cells by western blot. As shown in Figure 3, P16-INK4A expresses in all tested cell lines and its level is lower in KYSE-450 cells than in KYSE-150 cells. Based on Figure 1C result in this study, KYSE-150 cells seem to be more sensitive to JQ1-induced cell migration than KYSE-450 cells, indicating that ESCC cells with high level of P16-INK4A might be easier to occur cell migration when clinical application of BRD4 inhibitor or targeting BRD4.The relevance of P16-INK4A level with JQ1-induced cell migration should be further verified in the future. We are planning to expand this study on other type cancer cells and will observe whether the effect of BRD4 inhibition on cancer cell migration is P16-INK4A level/function-dependently different.

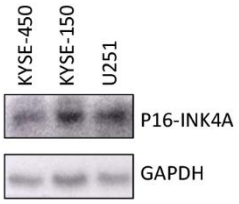

Figure 3 The expression levels of P16-INK4A protein in KYSE-450, KYSE-150 and U251 cells.

5. Discussion: specificity of BRD4 effects in ESCC should be addressed.

Reply: As described above, JQ1 not only inhibits human ESCC cell migration but also suppresses human glioma cell migration. On the basis of our observations, we speculate that the promoting effect of BRD4 inhibitor on cell migration is not ESCC cell specific. We have discussed this issue in the revised manuscript.

We are thankful to all the wonderful comments and please contact me if you have any other questions.

Sincerely,

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