Targeting BRD4: Potential therapeutic strategy for head and neck squamous cell carcinoma (Review)

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Abstract. As a member of BET (bromodomain and extra-terminal) protein family, BRD4 (bromodomain-containing protein 4) is a chromatin-associated protein that interacts with acetylated histones and actively recruits regulatory proteins, leading to the modulation of gene expression and chromatin remodeling. The cellular and epigenetic functions of BRD4 implicate normal development, fibrosis and inflammation. BRD4 has been suggested as a potential therapeutic target as it is often overexpressed and plays a critical role in regulating gene expression programs that drive tumor cell proliferation, survival, migration and drug resistance. To address the roles of BRD4 in cancer, several drugs that specifically target BRD4 have been developed. Inhibition of BRD4 has shown promising results in preclinical models, with several BRD4 inhibitors undergoing clinical trials for the treatment of various cancers. Head and neck squamous cell carcinoma (HNSCC), a heterogeneous group of cancers, remains a health challenge with a high incidence rate and poor prognosis. Conventional therapies for HNSCC often cause adverse effects to the patients. Targeting BRD4, therefore, represents a promising strategy to sensitize HNSCC to chemo- and radiotherapy allowing de-intensification of the current therapeutic regime and subsequent reduced side effects. However, further studies are required to fully understand the underlying mechanisms of action of BRD4 in HNSCC in order to determine the optimal dosing and administration of BRD4-targeted drugs for the treatment of patients with HNSCC.

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Contents

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent cancer globally, which arises from stratified mucosa of the mouth, trachea and larynx. Despite improved treatments, overall survival remains low, with more than 450,000 deaths in 2018 (1). There is a rise in annual incidence of oropharynx squamous cell carcinoma, a subtype of HNSCC, over the past decade, within the non-smokers, non-alcoholics and aged <50 years white male demographic group. This occurrence is associated with the human papillomavirus (HPV) infection, particularly HPV16, with the risk factor being an increase in sexual partners for oral or vaginal sex at a younger age (2).

Bromodomain protein 4 (BRD4) is one of the members of the bromodomain and extra-terminal (BET) family and a dual bromodomain protein consisting of two N-terminal bromodomains and an extra-terminal (ET) domain. BRD4 binds and acetylates lysine residues on target proteins including histones as a transcriptional and epigenetic regulator (3). BRD4 plays an important role in transcription, replication and DNA repair.

It also binds to non-histone proteins, DNA and RNA, contributing to development, tissue growth and various physiological processes (4). BRD4 is a crucial element in the regulation of cell cycle and mitosis which ensures the integrity of cell differentiation and development. The protein is also a predominant component of super-enhancers (SEs) associated with all active promoters and a significant proportion of active promoters in the genome including activation of genes involved in cell growth and cell cycle progression (5,6). The critical roles of BRD4 regarding transcriptional regulation are growth and cell division, metabolic processes, immune responses and embryo development regulation. In normal and transformed cells, BRD4 dysfunction results in pathogenesis and disease development in humans, such as prolonged inflammation, as the protein directly regulates the activity of NF-κB including inflammation, fibrosis, viral infections and neoplasia (7,8). Several studies have addressed the involvement of BRD4 in development of various tumors, where BET can promote aberrant expression of oncogenes such as c-Myc in acute lymphocytic leukemia and acute myeloid leukemia (AML) (9). The diverse role of BRD4 depends in several contexts on its interaction partners. It is considered that interfering with BET activity can reduce cancer cell proliferation and induce apoptosis (10). The most significant evidence for involvement of BRD4 in HNSCC carcinogenesis came from NUT carcinoma. Inhibition of the BRD4-NUT fusion gene on the BRD4 section using BETi (BET inhibitors) stalled the growth of NUT carcinoma cells (11). Therefore, targeting BRD4 through inhibition of BET protein has been explored as a therapeutic option for various cancers including HNSCC.

2. The normal functions of BRD4

BRD4 is an indispensable protein for cellular development. For example, bone marrow stem cells cannot differentiate into lymphoid stem cells without the presence of BRD4 (12). Moreover, the full expression of BRD4 is also essential for embryogenesis. A previous study conducted in mice reported that one allele of BRD4 was only enough to allow the embryonic stem cells to differentiate but insufficient for complete mouse development (13).

Post-translational modification of histone alters gene expression by regulating the chromatin landscape through changing the overall charge of the chromatin which recruits chromatin modifier enzymes (14). The epigenetic phenomenon is partly operated by BRD4 as the protein has histone acetyltransferase (HAT) and kinase activities phosphorylating serine2 residue of the RNA polymerase II carboxy-terminal domain. The binding of bromodomains to acetylated histone and lysine residues at the histone H3 site and H4 on chromatin regulates downstream gene expression. As BRD4 regulates chromatin remodeling by acetylating histone H3 Lys122, it causes instability and ejection of nucleosomes from chromatin as well as chromatin structural detachment; this leads to an increase in transcription. The resulting chromatin fragmentation permits DNA accessibility and allows access to transcriptional machinery (15-17). The perturbed chromatin structure and nucleosome remodeling at the promoters allow transcription factors as well as RNA Polymerase II to enter and start the transcription process (18). Furthermore, BRD4 coupling with RNA Polymerase II complex assists the complex to elongate through hyperacetylated nucleosomes by interacting with acetylated histones using bromodomains (19).

The HAT activity of BRD4 is responsible for a smooth transition from G1 to M phase of the cell cycle as it mediates transcription and pause-release. Similarly, the G2 to M phase transition has been known to be under the control of BRD4 via its interaction with a GAP protein, SPA-1. This again, relieves the block to cell cycle progression (20). Likewise, BRD4 controls the levels of Aurora B which is concentrated around the sites of attachment of chromosomes to spindle microtubules such as the centromeres or kinetochores and allows for chromosome segregation to occur appropriately (21). With low levels of BRD4, mitosis may become abnormal leading to increased incidence of lagging chromosomes, micronuclei and bridging chromosomes, eventually resulting in failed cytokinesis and multilobulated nuclei (16). As numerous genes regulated by BRD4 are involved in the processes of cell differentiation and development, dysregulation of BRD4 could become oncogenic which leads to pathogenesis of a wide variety of cancers (22).

3. Roles of BRD4 in tumor development

Aberrant expression or function of BRD4 is well-connected to oncogenic processes which includes HNSCC tumorigenesis (23). BRD4 has two well-structured N-terminal bromodomains (BD1 and BD2); in addition to BD1 and BD2, the molecular actions of BRD4 depend on the CK2-phosphorylated region, conserved ET domain and the distinct C-terminal motif. The regions are the interactive platform for recruiting chromatin and transcriptional regulators (24). BRD4 has three isoforms of different lengths but there are two main isoforms, BRD4 long (BRD4-L) and BRD4 short (BRD4-S). Evidence suggests that a disruption of the balance between the two BRD4 isoforms occurs in certain cancer types leading to substantial biological consequences (25). BRD4 and other BET proteins are often overexpressed in cancer and this leads to abnormal chromatin remodeling and tumorigenesis-mediated gene transcription. BRD4-mediated histone modifications regulate gene expression and maintain normal cellular homeostasis, which are vital for the cells (26). Studies conducted in human cancer types have shown that BRD4 overexpression is one of the reasons for oncogene amplification such as Myc, Notch3 and NRG1 leading to cancer progression (25,27). The progression of triple-negative breast cancer (TNBC) is also linked to increased phosphorylation of BRD4 in the acidic region due to decreased protein phosphatase 2A (PP2A) activity (28). These studies all point to BRD4 as a central protein for tumor development, specifically by inducing and maintaining the pool of cancer stem cells in squamous cell carcinoma including HNSCC (29).

Oncogenic mechanisms resulting from changes in genome structure may include mutations, copy number changes, or genome rearrangements. 'Oncogene addiction' is a mechanism used by cancer cells to maintain their unchecked proliferative needs (30). This is largely due to the functions of BET proteins, in addition to their role in transcriptional regulation by forming the Twist/BRD4/P-TEFb/RNA-Pol II complex which lead to stem cell-like properties and tumorigenicity (31). BRD4 is a key protein in numerous cancer hallmarks, it can stimulate

cancer cell proliferation through the functions of Jagged1 and Notch1 in breast cancer (32). It also controls oncogenic network gene expression by interacting with acetylated transcription factors including RELA, ER, p53 and twist (33). BET inhibitors have demonstrated remarkable anticancer effects for treatment by interfering with BRD4 expression or activity and effectively inhibit the progression of the cell cycle and induce apoptosis which reduces tumor cell proliferation and subsequent cancer development (34-36). Inhibition of BRD4 has revealed significant effect on sensitizing various tumor cell types to therapeutic agents including diffuse large B-cell lymphoma, neuroblastoma, lung cancer, NUT midline cancer and HNSCC (37-41). BRD4 has been linked with poor prognosis in a wide range of cancer patients including HNSCC (23,27). In addition to solid tumors, BRD4 inhibition has also been identified to be effective against hematological malignancies (42). The anticancer efficacy of BRD4 has been reported and clinical trials are ongoing (10,43). Incoming data from these studies will further validate the use of BRD4 inhibitor in antitumor therapy. However, it is critical to investigate whether targeting BRD4 is feasible for HNSCC treatment as there is a currently unsolved dilemma for the cancer as discussed below.

4. DNA damage repair and therapy resistance

Genetic mutations resulting from unrepaired DNA damage may increase the risk of precipitating genetic disorders and cancers. Although an isoform of BRD4 functions as an internal inhibitor of DNA damage response by remodeling the chromatin complex (44), the protein transcriptionally regulates DNA damage repair-related genes such as RAD51AP1 and TopBP1 as well as engages in double strand breaks (DSBs) through both non-homologous end joining (NHEJ) and homologous recombination (HR) pathways (45-48). BRD4 particularly involves in HR through direct contact with the SWI/SNF chromatin remodeling complex (49). It is worth noting that BET proteins inhibition itself can induce DNA damage potentially through deposition of R-loops leading to transcription-replication collision events (46). Additionally, BRD4 assists in maintaining genome stability through non-transcriptional functions such as DNA damage repair, checkpoint activation and telomere homeostasis (27). The use of BET inhibitors, namely JQ1 and AZD5153, has been revealed to prolong DNA DSBs and repress NHEJ-related genes XRCC4 and SHLD1 (50). JQ1 treatment leads to the substitution of BET proteins and transcription regulatory complexes from acetylated chromatin (51). JQ1 not only increases the damage level of DNA, but also attenuates DNA damage repair, particularly double strand break repair, which consequently sensitize the tumor cells to PARP inhibitor Olaparib (52). Additionally, JQ1 can inhibit the growth of ARID2-deficient hepatocellular carcinoma cells as well as induce apoptosis when ARID2-depleted through the aggravated DNA damage of DSBs (53).

BRD4 amplification has been shown as a prognostic factor in various cancer types such as ovarian, esophageal, non-small cell lung cancer and HNSCC (23,25,54,55). This could be due to numerous pro-survival functions of the protein including acetylation of histone H4 by DNA damage recruits BRD4 to stabilize the DNA repair complex (47). The multiple underlying roles of BRD4 in DNA damage repair is conceivably the major contributor in tumor cell resistance to therapy. For HNSCC, mutations in the DNA repair genes have enabled HNSCC to become resistant to therapy (56). Additionally, certain DNA damage repair genes appear to be upregulated including Ku80 and APEX1 and linked with patient prognosis (57,58). However, a recent study has indicated that a change in the expression of individual DNA repair proteins may not necessarily cause resistance to therapy. Rather, a balanced expression and coordination within the DNA repair signaling cascade is rather the actual cause of the resistance (59). Thus, targeting BRD4 protein which is upstream of DNA damage response may hypothetically benefit cancer patients, especially those with therapy-resistant HNSCC. The use of BRD4 inhibitor has been revealed to enhance the radiosensitivity of HNSCC as well as other tumors in pre-clinical models potentially through the upregulation of p21 and suppression of RAD51AP1 and Mcl-1 (41,60-63). Targeting the protein is also suggested to attenuate YAP, Myc-AP4 and E2F2 signaling which are often upregulated in various tumors (64-66). Degradation of BRD4 could cause a genome wide pausing of Pol II as BRD4-PTEFb is the main driving partner for phosphorylation of Pol II C-terminal domain and Pol II transcription (67,68). Similarly, BRD4 inhibition has been shown to sensitize colorectal tumor to doxorubicin as the protein is the cause of cisplatin resistance in bladder tumor through the Sonic hedgehog pathway (69,70). These accumulating data further have supported targeting the bromodomain protein as a therapeutic option to enhance the efficacy of current conventional therapies.

5. Epithelial mesenchymal transition (EMT) and cancer progression/aggressiveness

EMT remains a challenge in cancer treatment as the phenomenon provides not only an escape route with resistant features for cancer cells under therapeutic stress but also an opportunity for tumor expansion and metastasis. BRD4 is regarded as a key regulator of EMT as it governs key transcription factors that drive EMT particularly through the transcription of snail, both SNAI1 and SNAI2, as well as involves in TGF-β mediated EMT (71,72). Coupling between BRD4 and di-acetylated Twist was also shown to enhance downstream transcriptional targets of Twist for EMT (31,73). Overexpression of BRD4 enhances EMT and EMT is inhibited with reduced expression of BRD4 (74). There is a controversy whether the other BET proteins are involved in activation of EMT, namely BRD2 and BRD3; essentially, they are demonstrated to have a degree of control over EMT activation (75). Inhibition of BRD4 has frequently been demonstrated to suppress EMT through various mechanisms; for example, through activation of the NF-κB-NLRP3-caspase-1 pyroptosis signaling pathway in renal cell carcinoma (51), and inhibition of RelA-initiated TGF-β induced EMT via inflammatory tissue remodeling (76). BRD4 also regulates Jagged1 expression and Notch1 signaling for cancer cell dissemination (32). Treatment with BRD4 inhibitors has been identified to effectively suppress EMT-associated tumor invasion and metastasis through the regulation of key EMT proteins as well as attenuate the expression of MMP-2 and MMP-9, thus reducing HNSCC metastatic potential (77-80). Likewise, the activity of BRD4

in transitioning HNSCC cells to mesenchymal phenotype has equipped the cells to become cisplatin-resistant (81). These studies further emphasize the roles of BRD4 in tumor proliferation and expansion. The multiple functions of BRD4 on the development and expansion of HNSCC described are demonstrated in Fig. 1.

6. BRD4 in inflammation

Research involving BRD4 has evolved greatly as a result of its role in inflammation, which is associated with cancer through genomic instability, a cancer hallmark. Various studies have established that cancer is a disease that develops and progresses within inflammatory diseases including HNSCC (82). BRD4 enhances acetylation of RelA-K310ac which activates cyclin-dependent kinase 9 (CDK9), leading to the phosphorylation of RNA polymerase II to promote NF-κB gene transcription, thereby initiating the production of proteins responsible for inflammatory stimuli (83). Additionally, BRD4 regulates the expression of inflammatory genes through activation of enhancer RNAs or the Mnk2-eIF4E pathway-dependent translational regulation of IκBα synthesis in modulating inflammatory gene expression (84). There is also a direct association between BRD4 and the acetylated p65 subunit of NF-κB as well as the transcription factor of Nrf2, a key regulator of inflammation (85). Studies have investigated the efficiency of BRD4 inhibition of the inflammatory process. In primary human umbilical cord-derived vascular endothelial cells treated with TNF-α, JQ1 lessens the overexpression of FN1 induced by TNFα and could possibly slow down the progression of atherosclerosis (86). JQ1 has been shown to effectively protect colon-tight junctions from endotoxemia-induced inflammatory injury (87). In rat kidney triggered by Cadmium for inflammatory response, BRD4 inhibition reduced NF-κB nuclear translocation and its subsequent transcriptional activity (83). The use of I-BET, a BET inhibitor, can effectively inhibit pro-inflammatory protein production in lipopolysaccharide-activated macrophages (88). In primary and human bronchial epithelial cell lines, oxidative stress induced by IL-1β was significantly reduced by BRD4 inhibition (89). These studies have implicated that targeting BRD4 can subside inflammation in HNSCC and maybe beneficial to the patients as various studies have reported that inflammatory markers are prognostic factors for these patients (90-93). Thus, alleviating inflammation as a result of targeting BRD4 could prove to be useful in the treatment of HNSCC.

7. The relationship between BRD4 and HPV

Most common in the United States and other high-income countries, HPV-related HNSCC is becoming far more common than HPV-associated cervical cancer (94). Oncogenic HPV can be latent and cause malignant transformation years later; however, infection of high-risk HPV types can lead to pre-cancerous, in certain tissue, and cancer (95). A total of ~25% of all HNSCCs were positive for HPV-DNA with HPV-16 being the most prevalent subtype (96). There are 15 high-risk types of HPV, but the two most common ones, HPV16 and HPV18 accounting for ~72% of the total (97). The life cycle of HPV is highly dependent on the host cellular differentiation program. Although a receptor for HPV infection has not been recognized, it has been postulated to be heparan sulfate proteoglycan on the basal membrane (98). The role of viral protein E1 is unclear, whereas E2 is responsible for the transcription of E6 and E7 viral genes. In addition, the binding of HPV E2 protein to DNA is involved in viral DNA replication, transcription, genome maintenance and isolation (99). HPV E6/E7 expression is required for the binding of viral genome to DNA in the regions of genomic instability. This is followed by disruption of the E2 coding region and abnormal regulation of E6/E7 itself. Because of this, HPV can produce persistent infection (100). Degradation of p53 and pRb, by E6 and E7, respectively, contribute to cancer induced by this virus (101).

In HPV-associated tumors, BRD4 plays an important role in replication of HPV (17). The viral genome is attached to the mitotic chromosomes for segregation; BRD4 is used as a cellular adapter, where BRD4 typically interacts with the virus-encoded E2 protein to facilitate viral genome segregation (102,103). BRD4 and E2 form a complex between the viral genome and the host chromosomes to allow the viral genome insertion at fragile sites of the host genome (104). BRD4 is recognized as an atypical chromatin binding factor that binds to chromosomes throughout mitosis, known as MCAP (mitotic chromosome-associated protein) and is expressed as a mitotic bookmark. As transcriptional regulation is a fundamental role of BRD4, it is vital for several E2 functions and stability. Disruption of the interaction between BRD4 and E2 inhibits E2-mediated transactivation (105). In conjunction with E2, BRD4 is required to suppress the transcription process in early viral promoter, an essential process during the early gene expression in order to maintain the infection in the basal cells, in which the copy number of the viral genome remains very low (106). Phosphorylation of BRD4 regulated by Casein kinase II and PP2A is essential for the binding of BRD4 to acetylated chromatin and recruiting major transcription factors including p53, AP-1 and NF-kB to control the viral transcription program (107). The role of BRD4 is evidently important in regulating HPV transcription particularly in the early stage of viral transcription. Treatment with BETi has been shown to reduce the viral transcription in a HPV11 infected model (108). A combination of BET inhibitor and HDAC6 inhibitor has demonstrated significant synergistic effects against HPV-positive and HPV-negative in HNSCC cells (77). Similarly, it has beem reported by the authors that BRD4 inhibitor is effective in reducing HPV E6/E7 transcription in HPV-associated HNSCC cell lines (37). It is also worth noting that inhibition of BRD4 also offers antiviral activities by decompacting chromatin structure and activating DNA damage-dependent immune responses which attenuates viral attachment to the host chromosome and subsequently improves host resistance to viral infection (109). Thus, BRD4 inhibition is potentially an effective approach against viruses-associated malignancies (110).

8. BRD4 roles and therapeutic approaches for HNSCC

For primary HNSCC, surgical resection of the tumor and lymph node followed by radiotherapy with or without platinum-based chemotherapy or definitive concurrent chemoradiation therapy is the main modality for treating the patients (111). Cisplatin is often the chemo-reagent for the course; however, significant acute and late toxicity is

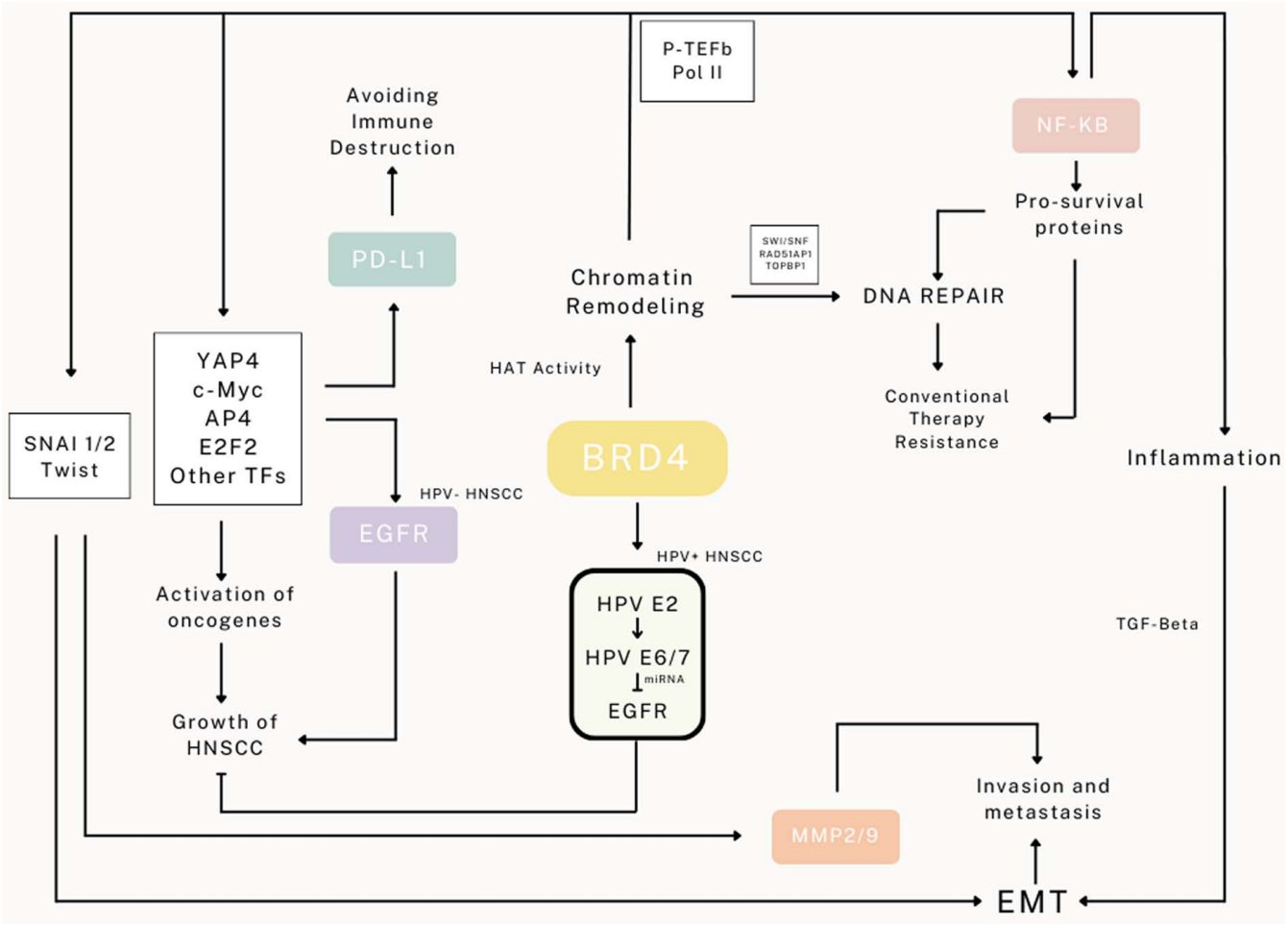

Figure 1. The involvement of BRD4 functions in HNSCC. BRD4 is involved in inflammation, therapy resistance and DNA repair partly through NF-κB. The protein regulates the transcription of c-Myc and HPV E2 which permit oncogenesis. BRD4 is associated with tumor aggressiveness and metastasis through EMT governing the transcription of key EMT genes. BRD4, bromodomain-containing protein 4, HNSCC, head and neck squamous cell carcinoma; EMT, epithelial mesenchymal transition; PD-L1, programmed death-ligand 1.

often observed (112). Thus, deintensification approach has been trialed using cetuximab to target epidermal growth factor receptor (EGFR), replacing cisplatin for those with HPV-positive HNSCC. However, the patients receiving cetuximab appear to be at a higher risk of death and relapse of the disease than those receiving cisplatin (113,114). Despite EGFR upregulation is an acknowledged biomarker suggesting treatment resistance and aggressiveness in HNSCC (115), targeting EGFR has shown significant benefits for HPV-negative HNSCC (116). However, inhibition of the receptor in HPV-associated HNSCC leads to lesser therapeutic outcomes suggesting that EGFR plays opposing roles in the two HNSCC subtypes (117). Increasing evidence has demonstrated that cetuximab may not be the best course for HPV-positive HNSCC therapy (118,119). In HPV-positive HNSCC cells, overexpression of EGFR suppresses cellular proliferation and increases radiosensitivity through inhibition of BRD4 via miR-9-5p and subsequently reduced HPV E6/E7 transcription (37). Therefore, targeting EGFR may not be the best course of therapy for HPV-positive HNSCC, but targeting specific signaling pathways such as BRD4 could provide a preferable new treatment to improve the therapeutic outcome of HNSCC (120).

BRD4 has been clinically linked to several oncogenes, such as activating Myc in leukemia and lymphoma (121). It has also been observed that BRD4 protein and its mRNA levels are abnormally regulated in HNSCC samples, correlated with tumor features such as size, proliferation and advanced disease degree (23). A previous study in HNSCC reported that BRD4 overexpression decreases the mRNA stability of cyclin-dependent kinase inhibitor 1B (p27), and the protein p27 is responsible for inhibiting tumor progression (122). The protein can also act as a pro-oncogene that accelerates tumor growth and metastasis as a critical part of SEs (123). BRD4 has thus been identified as a prognostic biomarker of HNSCC (23).

Inhibition of BRD4 using JQ1 has been demonstrated to induce senescence in head and neck tumor cells through downregulation of acetylated histone H4 and phosphorylated SIRT1(ser47) leading to p21 and p16ink4 accumulation (124). Treatment with the inhibitor also blocks SEs, decreases TP63 expression in HNSCC, and effectively eliminates both cancer stem cells and lymph node metastasis (125). Additionally, BRD4 is a regulator of JOSD1, a protein linked to poor prognosis in patients with HNSCC. JQ1 treatment has been identified to downregulate both the JOSD1 protein and mRNA expression. Overexpression of the protein indicates

a poor clinical prognosis for patients with HNSCC (126). Similarly, cooperation between YAP1 and BRD4, which can be attenuated by JQ1 treatment, enhances HNSCC tumorigenesis (127). Treatment with the inhibitor has been also shown to overcome cetuximab resistance in HPV-negative subtype of HNSCC (128). These studies have accumulated evidence in favor of the use of BRD4 inhibitor as a part of HNSCC therapy in the near future to resolve the dilemma of targeting EGFR in HPV-associated HNSCC, as demonstrated in Fig. 2.

9. BRD4 and immune response

Tumor cells often modulate the expression of genes or immune signaling pathways to avoid immune recognition and promote tumor growth and metastasis (129). Therefore, it is critically important to recognize their interaction with immunologic cells in order to stratify toward immunotherapy. Immune cell infiltration into tumor tissue, particularly for cytotoxic T lymphocytes and natural killer cells have been closely investigated in recent years. The roles of BRD4 regarding immune response to tumor have been investigated with BRD4 expression being associated with levels of infiltrating monocytes, tumor-associated macrophages, M1/M2 macrophages and T cells (Th1/Th2/Treg) in breast cancer (130). In hepatocellular carcinoma, expression of BRD4 mRNA is elevated and correlated with immune infiltrating levels of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils and dendritic cells (131). BRD4 expression is also connected with low infiltration of T-bet^+ tumor-infiltrating T lymphocytes leading to poorer prognosis potentially through activation of Jagged1 signaling pathways (132). BRD4 regulates programmed death-ligand 1 (PD-L1) expression through c-Myc implicating that targeting BRD4 can influence immune system against tumor cells (133). Suppression of BRD4 leads to down-regulation of PD-L1 in TNBC, thus potentially permitting an improved outcome with immunotherapy approach (134). BRD4 expression, above the other BET proteins, is the most negatively correlated with immune checkpoint as well as abundance of macrophage, neutrophil and $CD8^+$ T-cell in glioblastoma multiforme (135). These studies have all designated BRD4 as a prognostic marker for patient survival further highlighting the bromodomain protein as a therapeutic target. Furthermore, for HNSCC, the expression of PD-L1 in HNSCC cell lines could be reduced by JQ1 or MZ1 treatment (136). Likewise, suppression of the BET protein could enhance antitumor immunity through the induction of MHC class I expression and consequently improve the efficacy of anti-PD-1 immunotherapy in an *in vivo* model (137).

Although the exact mechanism on how BRD4 mediates tumor microenvironment and immune infiltration needs further elucidation, BRD4 is involved in the acetylation of lysine-310 of the RelA NF-κB subunit which activates the transcription factor and modulates proinflammatory cytokines as well as Th17 immune response (138). The epigenetic regulator has been shown to be responsible for the expression of a cohort of immunosuppressive genes including PD-L1, PD-L2, HVEM, GAL9, IL6, IL8, CSF2, BIRC3, IDO1 and IL1B (139). BRD4 is also suggested to be the protein responsible for immunosuppressive M2 macrophage polarization (140,141). Collectively, these studies have provided evidence that targeting BRD4 may shift the landscape of tumor microenvironment for immunotherapy and antitumor immune response which could be useful for the treatment of HNSCC as well as other solid tumors. It is enticing to explore the interaction between BETi and immunotherapeutic agents such as nivolumab and pembrolizumab which have been approved for HNSCC therapy. The co-administration between BETi and immunotherapy could lead to an effective therapy against HNSCC.

10. Resistance to BET inhibition

Despite the promise of BRD4 inhibition in cancer therapy mentioned, it has been suggested that the efficacy of BRD4 inhibition as a monotherapy could be transient and moderate (123). Several studies have demonstrated that tumor cells may develop resistance after a prolonged use of JQ1 due to the rewiring of proteins involved in transcriptional regulation which also affect other chromatin-targeted therapies. For example, in AML, WNT/β-catenin signaling pathway is shown as the primary and acquired driver for resistance to BETi (142,143). In lung adenocarcinoma, BET inhibition is effective in blocking cell growth through FOS-like 1 (FOSL1) suppression; however, resistance to JQ1 occurs independently of its effect on FOSL1 or Myc expression. Phosphorylation of BRD4 by casein kinase 2 (CK2) is suggested as a cause of BETi resistance (144). DUB3, which is upregulated by JQ1 treatment, deubiquitinates and stabilizes BRD4 causing prostate cancer to become resistant to BETi (145). Similarly, JQ1 resistance was demonstrated to be due to the loss of BRD4/FOXD3/miR-548d-3p axis which is compensated by JunD/RSK3 signaling which essentially builds up BETi resistance in basal-like breast cancer (146). For TNBC, the cells can rapidly develop resistance due to various mechanisms, including changes in signaling pathways involving ZNF33A upregulation, deletion of SNF/SWI complex components as well as ubiquitination-related genes such as SPOP, UBE2M, CUL3 and USP14 (147,148). In ovarian cancer cell lines, autophagy (shown by increased expression of ATG5 and Beclin1) induced by inactivation of Akt (Ser473)/mTOR (Ser2448) pathway, is linked with resistance to BETi possibly as a way to bypass BET inhibition (149). Thus, it is essential to note that resistance to JQ1 has distinct mechanisms depending on cancer types; however, increase in Myc expression has been pinpointed as common cause of resistance to BETi (147,150,151). BRD4 stabilization and its subsequent activation of AKT-mTORC1 activation has also been described as another route of BETi resistance (152). Therefore, the issue of resistance to BET inhibition should be closely investigated especially when BET inhibition is applied particularly as a monotherapy. The use of BRD4 inhibition as a part of combination therapy could be a more viable option considering this matter.

11. Clinical response to BRD4 targeting and adverse events

Currently, no BETi has been approved by the US FDA for the use of cancer; however, there are a number of phase I clinical studies which have provided initial information regarding the safety of BETi in patients. As shown in Table I, BETi appears to be safe in most patients with the prevalent treatment emergent adverse events (TEAEs) including thrombocytopenia, diarrhea, nausea, anorexia, vomiting, fatigue and anemia. The most significant dose-limiting toxicities are thrombocytopenia and

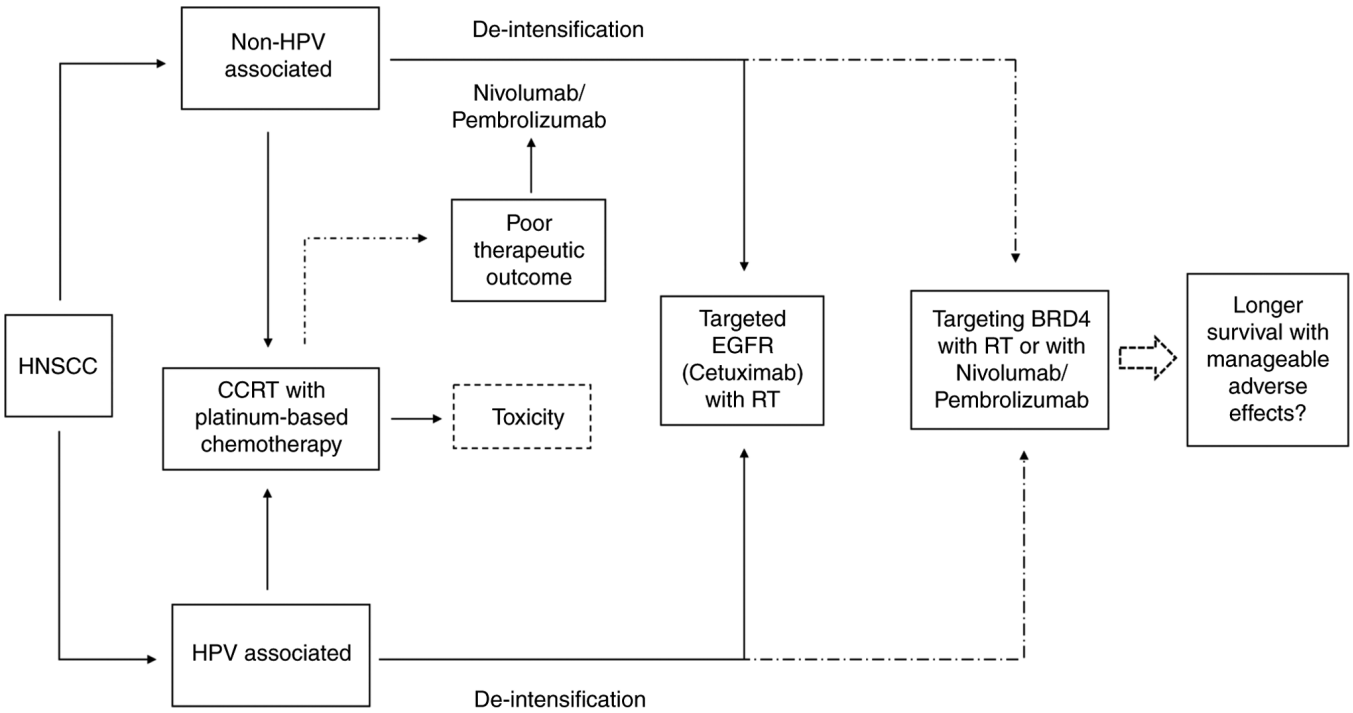

Figure 2. The current paradigm of HNSCC therapy. HPV status should be considered as a factor deciding the course of therapy. Targeting BRD4 replacing EGFR could possibly allow de-intensification of the conventional therapy applied for HNSCC therapy without compromising the treatment outcome. HNSCC, head and neck squamous cell carcinoma; BRD4, bromodomain-containing protein 4; HPV, human papillomavirus; CCRT, concurrent chemoradiation therapy; RT, radiotherapy.

fatigue. All studies have concluded that BETi was largely tolerable by the patients; thus, applying BETi for deintensification of HNSCC therapy could be viable although more data should be collected from further clinical studies especially for patients with HNSCC. Regarding the potential efficacy against HNSCC, it is premature to conclude the antitumor efficacy of BETi as the majority of these clinical studies have been conducted in patients with hematologic malignancies. However, a few studies in solid cancers have mentioned that the patients had achieved longer progression-free survival with 95% confidence intervals of [1.8-1.9] and [4.6-12.9] (153,154). Despite the promising safety of BETi in patients, future clinical studies should proceed with care and prepare to address the TEAEs which are likely to emerge.

12. Toxicity of BET inhibition

BET inhibitors have been tested and assessed in both *in vivo* models and human clinical trials for their safety and efficacy in cancer therapy. As BET family proteins play critical roles in regulating multiple cellular functions, it is expected that BET inhibition would have adverse effects. These side effects have been observed in various occasions in animal models following the tests to the animals, as listed in Table II. Additionally, the widely used *in vitro* inhibitor JQ1 has failed to advance to human clinical trial due to its poor pharmacokinetic profile (34). Another adverse effect which has been observed in the animal models is thrombocytopenia (155). Consistently, a systematic review of various BET inhibitors administered to treat hematological malignancies and solid tumors indicated that all BET inhibitor leads to exposure-dependent thrombocytopenia (43). Thus, the issue of adverse effects should be closely monitored for patients receiving BET inhibitor as a part of their cancer therapy courses particularly for those with HNSCC which could be affected by toxicities to the surrounding organs in the head and neck region.

In order to limit the toxicity of BETi, an alternative approach for precise delivery of BRD4 inhibitors or other targeting molecules is engineered exosome. The idea of exploiting exosome as a drug delivery system has become popular as it can surpass barrier created by tumor microenvironment and can be equipped with targeting properties. Small molecule drugs such as paclitaxel and curcumin have been delivered to specific target cells (156). Delivery of microRNAs targeting BRD4 could perhaps further alleviate the adverse effects shown by several BETi as the formation and delivery of exosomal microRNAs is becoming more practical (157). The precision in drug delivery will surely offer a more endurable therapy for the patients.

13. Future perspective

A number of BET inhibitors have shown great potential to be effective for cancer therapy which could enhance the efficacy of chemo-, radio- and immunotherapy against HNSCC. As a chromatin-targeted therapy, BET/BRD4 inhibitor could be a viable candidate for replacing the EGFR inhibitor knowing that it could be effective against HNSCC regardless of its HPV association, as cetuximab may not provide the best outcome for the HPV-associated subtype of head and neck cancer. Another potential role which targeting BRD4 may come into play is the de-escalation of the current HNSCC therapy regimens which are facing a challenge in terms of the side effects. This could

Table I. Clinical studies of BET inhibitors and the observed adverse events in the patients.

BET inhibitor	Target	Disease	Phase	Adverse effects	(Refs.)
ABBV-075	BRD2/3/4, BRDT	Uveal melanoma, breast cancer, pancreatic, HNSCC, CRPC, and others	I	- TEAEs were dysgeusia, thrombocytopenia, fatigue, and nausea - TEAEs (Grade 3/4) were thrombocytopenia and anemia - DLT included thrombocytopenia, gastrointestinal bleed, hypertension, fatigue, decreased appetite, and aspartate aminotransferase elevation	(153)
FT-1101	BRD2/3/4, BRDT	AML, MDS, NHL	I	- TEAEs (all grades) were diarrhea, fatigue, dyspnea, nausea, anemia, and platelet count decreased - AML/MDS pts had diarrhea, nausea or pleural effusion, cough, decreased appetite, or dyspnea - Severe (≥ grade 3) TEAEs were anemia, decreased platelets, pneumonia, sepsis, febrile neutropenia, and disease progression	(160)
GSK525762/ I-BET762	BRD2/3/4, BRDT	CRC, NUT carcinoma, and other solid tumors	I	- TEAEs (all grades) were thrombocytopenia, diarrhea, nausea, vomiting, anemia, decreased appetite, dysgeusia, and fatigue	(161)
		R/R AML, AML after MDS, new AML	I/II	- TEAEs were dysgeusia, diarrhea, nausea, and elevated bilirubin - DLTs at 100 mg: grade 3 diarrhea - DLTs at 120 mg: grade 3 ejection fraction decrease - TEAEs (Grade 3/4) were diarrhea, febrile neutropenia, thrombocytopenia, hyperglycemia, and fatigue/asthenia	(162)
INCB057643	BRD2/3/4, BRDT	Any advanced/recurrent malignancy Prostate cancer, colorectal cancer, breast cancer, ovarian cancer, lymphoma, AML, pancreatic cancer, glioblastoma, MDS, and others	I/II	- TEAEs (All grades) were Nausea, thrombocytopenia, fatigue, decreased appetite, diarrhea, dysgeusia, anemia, hyperglycemia	(163)
		R/R MF, other advanced myeloid neoplasms	I	- TEAEs were thrombocytopenia, dysgeusia, nausea, anemia, blood bilirubin increased, ejection fraction decreased	(164)
ZEN003694	BRD2/3/4, BRDT	Metastatic CRPC	Ib/IIa	- Toxicities grade ≥ 3 included: nausea, thrombocytopenia, anemia, fatigue, and hypophosphatemia - TEAEs were visual symptoms	(154)
ODM207	BRD2/3/4, BRDT	CRPC, melanoma, NMC, ER^+BC, and Solid tumors	I	- DLT was intolerable fatigue - Common TEAEs were thrombocytopenia, asthenia, nausea, anorexia, diarrhea, fatigue, and vomiting	(165)
OTX015	BRD2/3/4	AML, ALL, RA with a large number of blasts	I	- Non-cumulative grade 1-2 gastrointestinal events (diarrhea, dysgueusia, abdominal pain, nausea, anorexia), hyperglycemia, coagulation factor VII decrease, and direct bilirubin increase - DLT was diarrhea, anorexia/fatigue	(166)
		Acute leukemia, MDS	I	- DLT was G3 diarrhea, G3 fatigue. Diarrhea, fatigue, and rash limited compliance	(167)

Table I. Continued.

BET inhibitor	Target	Disease	Phase	Adverse effects	(Refs.)
		Lymphoma, myeloma	I	- DLTs: G4 thrombocytopenia, G4 neutropenia, G3 hyponatremia - Other toxicity: Grade 1-2 events: gastrointestinal/diarrhea, nausea, fatigue, dysgeusia, anemia	(167)
		NMC, CRPC, NSCLC	Ib	- DLTs at 80 mg once daily: grade 3 thrombocytopenia, ALT/hyperbilirubinemia - DLTs at 100 mg once daily: grade 2 anorexia and nausea, grade 4 thrombocytopenia - TEAEs were diarrhea, nausea, anorexia, vomiting, thrombocytopenia	(168)
CC-90010	BRD2/4	Solid tumors, NHL	I	- TRAEs (Grade 3/4) were thrombocytopenia, anemia, and fatigue - TRAEs (Grade 4) thrombocytopenia associated with grade 3 skin hemorrhage - TEAEs (Grades ≤3) thrombocytopenia, hyperglycemia, and asthenia - TEAEs were nausea/vomiting, fatigue/asthenia, and thrombocytopenia	(169)
CPI-0610	BRD4	R/R Lymphoma	I	- Primary toxicity: Thrombocytopenia, Grade 3 diarrhea, rash, neutropenia	(170)
		Myelofibrosis	II	- TEAE (All grade): diarrhea, nausea, cough, and upper respiratory tract infection - TEAEs (≥3 Grade) were anemia and thrombocytopenia	(171)
PLX51107	BRD4	Solid tumors, lymphoma, AML, MDS	I	- Nonhematologic toxicities were febrile neutropenia and pneumonia in 12 patients each; 6 patients had severe hyperbilirubinemia - TEAEs (Grade 1-2) were fatigue, vomiting, diarrhea, nausea, bilirubin increase, and INR increase - TEAEs were G3 nausea, G2 vomiting, and G2 kidney injury - Uveal melanoma, sarcoma, NSCLC, breast cancer and CRPC, and other solid tumors	(172)
			II	- The most common toxicity was grade1-2 fatigue, vomiting, diarrhea, nausea, bilirubin increase, and INR increase - TEAEs: G3 nausea, and G2 kidney injury - DLTs were G3 thrombocytopenia, G3 nausea, G2 kidney injury	(173)
RO6870810/ TEN-010	BRD4	MDS, AML	I	- TEAEs were fatigue, decreased appetite and injection-site erythema, injection-site pain, nausea, fatigue, thrombocytopenia, anemia, and hyperbilirubinemia	(174)
		NC, DLBCL	I	- TEAEs (All grades) were fatigue, decreased appetite and injection-site erythema, injection-site pain and nausea - TEAEs (grade 3/4) were fatigue, thrombocytopenia, anaemia, and hyperbilirubinemia	(175)
		NMC	I	- Nonhematologic toxicities were Irritation at injection site, hyperbilirubinemia, and anorexia	(170)

Table I. Continued.

BET inhibitor	Target	Disease	Phase	Adverse effects	(Refs.)
AZD5153	BRD4	R/R malignant solid tumor and lymphoma	I	- TEAEs (Grade ≥3) were thrombocytopenia, fatigue, anemia, diarrhea, and platelet count decreased. - DLTs were thrombocytopenia and diarrhea with herpetic rash	(176)
BMS986158	BRD4	Advanced cancer	I/IIa	- TEAEs (Grade 3-4) were diarrhea, thrombocytopenia, fatigue, nausea, anemia, and vomiting - DLT was Grade 4 thrombocytopenia	(177)
GSK3358699	Pan-BET	Healthy subjects	N/A	- Headache, non-sustained ventricular tachycardia, tachycardia, ventricular extrasystoles, and atrial fibrillation	(178)

R/R, relapsed/refractory; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; NSCLC, non-small cell lung cancer; CRPC, castration-resistant prostate cancer; RA, refractory anemia; CRC, colorectal cancer; NUT, Nuclear protein of the testis; NMC, NUT midline carcinoma; ER + BC, estrogen receptor-positive breast cancer, Head and neck squamous cell carcinoma; MF, Myelofibrosis; NC, nuclear protein of the testis carcinoma; DLBCL, diffuse large B-cell lymphoma; TEAEs, Treatment emergent adverse events; DLTs, Dose-limiting toxicities.

Table II. Summary of the results from pre-clinical models inhibiting BRD4 using three different BET inhibitors, JQ1, I-BET-151 and ABBV-075.

BET inhibitor	BET targets	Disease	Model	Toxicity in clinical or model studies
JQ1	BRD2/3/4	Acute myeloid leukemia (143)	Mouse model	Model studies
		Triple-negative breast cancer (148)	Patient-derived xenografts	- Dosage-dependent toxicity and long-term JQ1 treatment have been shown to affect resistant cells (143)
		Multiple myeloma (180)	Mouse xenograft model	
		Normal cells and mesenchymal stem cells: Neuronal derivatives (181)	*In vitro*	Clinical studies - JQ1 has not been tested in clinical trials due to its poor pharmacokinetic profile, low oral bioavailability, and the need for the drug to be administered twice per day (34)
		Pancreatic cancer (182)	*In vitro* and *in vivo*	- High doses of JQ1 have been associated with potential toxicity (179)
I-BET-151	BRD2/3/4/9	Cardiac	*In vivo* (mouse and rat)	- I-BET-151 exhibits a dose-dependent reduction in the respiratory activity of cardiac mitochondria, related cardiotoxicity (183)
				- Cytotoxicity arises from disrupting fundamental cell processes such as cell growth and the progression of the cell cycle that occurs in all dividing cells (184)
ABBV-075	BRD4	Prostate cancer	Rat model	Model studies - Reduction in platelets and loss of goblet cells (155)

BRD4, bromodomain-containing protein 4; BET, bromodomain and extra-terminal.

allow lesser adverse effects to the patients which typically affect the patients' quality of life. A clinical trial proving the efficacy of BRD4/BET inhibitor for the treatment of HNSCC is also desirable in order to demonstrate its clinical application in addition to its potential shown *in vitro* and *in vivo* models. In addition, the combination between BRD4 and other inhibitors should be considered. For example, BRD4 inhibitor in combination with suberoylanilide hydroxamic acid as a histone deacetylase inhibitor have been tested and exhibited promising results in certain tumors (158,159). This approach could further expand therapeutic options but may also need to proceed with caution due to adverse effects of such inhibitions. The investigation concerning immunological effects of BET inhibition should also be considered to evaluate the applicability of targeting BRD4 in head and neck cancer therapy.

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Authors' contributions

NW, WK, NA and TK wrote the first draft of the manuscript. VY revised the manuscript and generated all figures and tables. DN conceptualized the study and critically revised the manuscript. MT provided guidance and edited the manuscript prior to submission. All authors read and approved the final manuscript. Data authentication is not applicable.

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Competing interests

The authors declare no competing interests.

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