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LETTER TO THE EDITOR

The oncogenomic function of androgen receptor in esophageal squamous cell carcinoma is directed by GATA3

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Dear Editor,

Esophageal carcinoma (EC) is the 6th leading cause of cancer death worldwide. In China, esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of EC and the 4th leading cause of death from cancer.[1,2] While esophagectomy has been central to the standard of care for localized ESCC, relapse often occurs rapidly. For advanced ESCC, multimodality therapies incorporating systemic chemotherapies and/or radiotherapy have yielded limited clinical benefit. Despite extensive research efforts, no targeted therapies have yet been approved for the treatment of advanced ESCC. Interestingly, ESCC is strongly characterized by a male-predominant propensity, as both incidence and mortality rate are 2–3-fold higher in males than in females.[1] Previous studies have revealed a possible association between androgen receptor (AR) signaling and male ESCC.[3,4] AR is a ligand-dependent transcription factor that regulates target gene expression through binding to androgen-responsive elements (AREs) in the presence of androgens. Conversely, AR antagonists (e.g., enzalutamide) compete with androgens to bind AR and inhibit its binding to AREs.[5,6] To date, while the genomic function of AR has been extensively studied in prostate cancer,[5,7,8] it remains unknown how AR exerts its oncogenic functions in ESCC at a genome-wide scale. Addressing this question is of high clinical relevance as it will establish a mechanistic basis for a promising therapeutic strategy targeting the AR transcription axis for ESCC patients.

We first analyzed the expression of AR in a cohort of 253 ESCC patients. High AR nuclear staining was observed in ESCC cells from > 45% of patients. High expression of AR correlated strongly with shorter overall survival and disease-free survival (Fig. 1a, b; Supplementary information, Fig. S1a). To study the putative oncogenic function of AR in ESCC, we used two male ESCC patient-derived, AR-expressing cell lines, KYSE410 and ZEC014. Treating KYSE410 and ZEC014 cells with AR agonists (dihydrotestosterone (DHT) or R1881) significantly promoted cell proliferation and survival, whereas AR antagonist (enzalutamide/XTANDI®) treatment counteracted androgen function and resulted in growth inhibition in both cell lines (Supplementary information, Figs. S1b–h, S2a–c). Consistent with the results from pharmacological inhibition of AR, genetic knockdown of AR significantly decreased cell growth of both KYSE410 and ZEC014 cells (Supplementary information, Fig. S2d, e). Importantly, AR-depleted KYSE410 cells exhibited impaired hormone responsiveness in cell proliferation compared to control cells (Fig. 1c), suggesting that AR is required for hormone-dependent growth in ESCC.

To elucidate the molecular mechanisms underlying AR-driven ESCC growth, we first performed AR ChIP-seq to define the AR cistrome in KYSE410 cells. We identified 714 enriched AR binding sites and 684 depleted AR binding sites in the R1881-treated condition relative to the vehicle control. Motif analysis revealed that a full-length ARE was significantly enriched among R1881-induced but not R1881-repressed AR binding sites (Fig. 1d), indicating that androgen-induced sites are directly mediated by AR binding to DNA. This finding was consistently validated in randomly selected R1881-induced AR binding regions (Supplementary information, Fig. S3a). RNA-seq analysis of cells treated with R1881 for 4 h or 24 h revealed that 65.8% and 57.9% of differentially expressed genes were downregulated by R1881 at these two time points, suggesting that androgen mainly represses gene expression in ESCC cells (Fig. 1e). qRT-PCR analysis of randomly selected genes validated the RNA-seq analysis (Supplementary information, Fig. S3b, c). To integrate the AR cistrome with hormone-regulated gene expression, we performed the Genomic Regions Enrichment of Annotations Tool (GREAT) (Supplementary information, Fig. S3d) and Gene Ontology (GO) term enrichment analyses. This revealed that genes associated with both gained AR binding and downregulation in response to R1881 treatment were involved in "Esophageal Neoplasm and Hormone-dependent Neoplasm". In contrast, genes associated with lost AR binding and upregulation in response to R1881 treatment were not enriched in any cancer-relevant pathways (Supplementary information, Fig. S3e, f). Our data suggest that androgen-induced direct AR binding may repress the transcription of cancer-related genes to promote ESCC growth.

Included among AR-downregulated genes were *DUSP4* and *FOSB*, for which lower expression was associated with worse clinical ESCC patient outcomes (Supplementary information, Fig. S4a–d). To characterize AR-mediated transcriptional repression of the clinically relevant *DUSP4* and *FOSB* genes, we first measured expression of these two genes in KYSE410 cells treated with R1881 or enzalutamide. R1881 exposure significantly suppressed expression of both genes, whereas enzalutamide treatment significantly elevated their expression (Supplementary information, Fig. S4e). We next performed AR ChIP assays to validate R1881-induced AR binding sites −33 kb and −1.2 kb from the transcription start sites (TSS) of *DUSP4* and *FOSB*, respectively. Treatment with R1881 but not enzalutamide significantly increased AR binding to the gene regulatory region of each locus (Supplementary information, Fig. S4f), while R1881-induced AR binding was abolished by AR silencing (Supplementary information, Fig. S5a, b). Furthermore, AR ablation impaired transcription repression of these two genes upon R1881 treatment, indicating that AR mediates androgen-induced transcription repression (Supplementary information, Fig. S5c, d). We next investigated the functions of these two genes using a dox-inducible Tet-On system, showing that dox-induced overexpression of *DUSP4* or *FOSB* significantly repressed growth of both KYSE410 and ZEC014 cells (Supplementary information, Fig. S5e–j). Additionally, the upregulation of *DUSP4* or *FOSB* and the growth inhibitory effects observed following AR depletion were abrogated by additional silencing of *DUSP4* or *FOSB* (Supplementary information, Fig. S5k–m).

Interestingly, analysis of potential collaborating transcription factor motifs within AR direct binding regions revealed that the GATA3 motif was significantly enriched (Fig. 1d). The combined presence of both ARE and GATA3 motifs was detected in 30.53%

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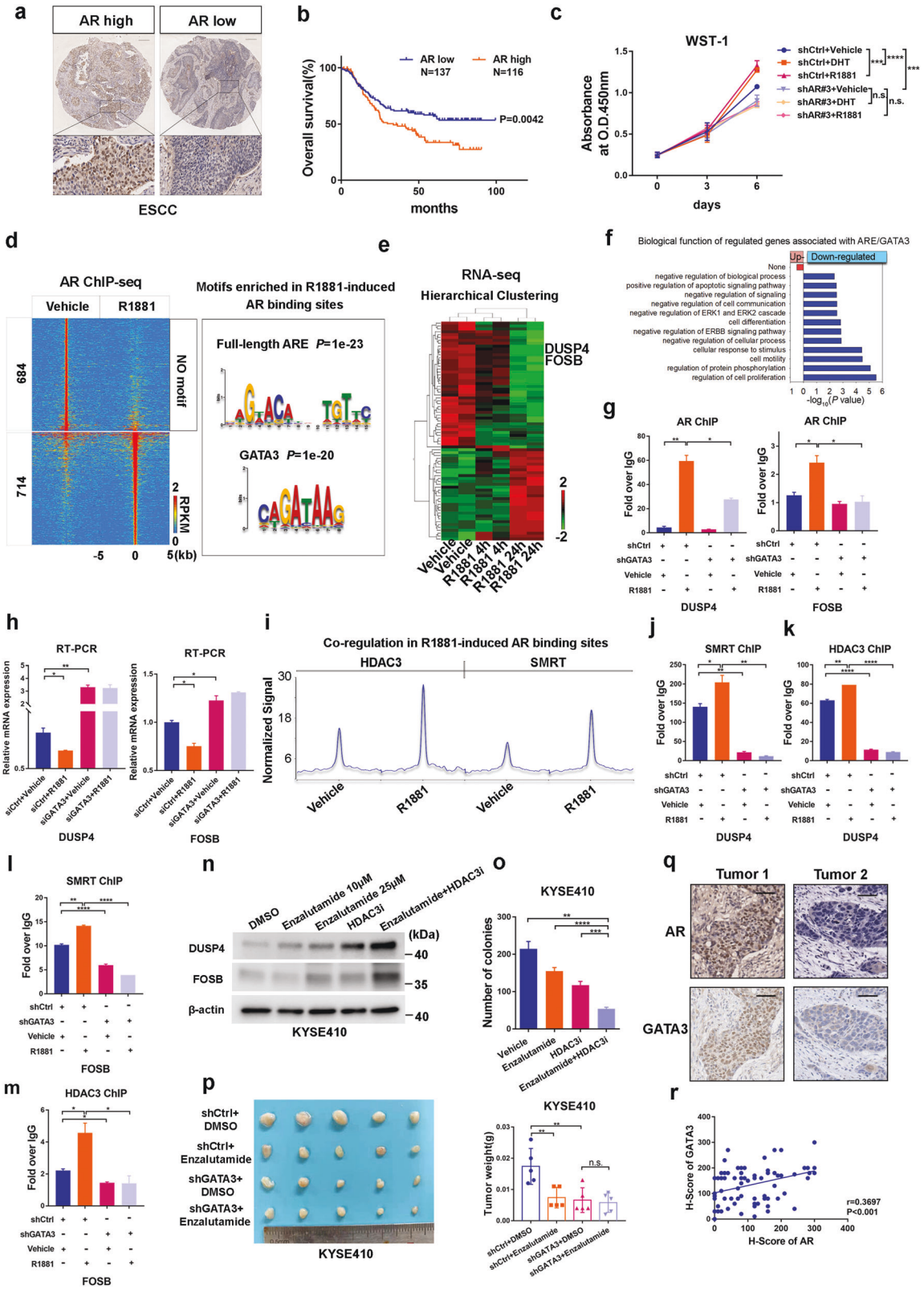

of androgen-induced AR-binding sites, which was the largest proportion of motif features compared to the occurrence of ARE only (19.47%) and GATA3 motif only (29.41%) (Supplementary information, Fig. S6a). The occurrence of the GATA3 motif in most AR binding sites (59.94%) suggests that the GATA3 transcription factor may play important and collaborative roles in AR genomic functions in ESCC cells. Notably, androgen-downregulated genes associated with the ARE/GATA3 motif were functionally enriched in cell proliferation and negative regulation of several oncogenic processes (e.g., the ERK and ERBB pathways) (Fig. 1f). Consistently,

Fig. 1 The transcriptional repression program of AR in ESCC is directed by GATA3. a Immunohistochemistry staining of ESCC tissues with AR antibody (ab105225). Representative images are shown. Scale bar, 200 μm. **b** Kaplan–Meier analysis of overall survival of ESCC patients grouped by low expression or high expression of AR. *P* values were determined by log-rank test. **c** The AR-depleted KYSE410 cells or control cells were cultured in phenol red-free RPMI 1640 medium with 5% charcoal-stripped FBS and then treated with DHT (100 nM) or R1881 (10 nM) for the indicated days. WST-1 assay was performed to evaluate the cell proliferation of the indicated groups. **d** Heatmap showing AR ChIP-seq signal intensity in KYSE410 cells (left). Consensus motif identified in the AR binding sites (right). **e** Heatmap of differentially expressed genes after R1881 treatment at the indicated time points. Differentially expressed genes across all samples after treatment with FDR < 0.05 were used in hierarchical clustering. The scale bar is shown with the minimum expression value for each gene in green and the maximum value in red. **f** Pathway analysis of regulated genes associated with ARE/GATA3 motif. **g** The GATA3-depleted or control KYSE410 cells were treated with vehicle or R1881 (10 nM) for 4 h. Standard ChIP was performed to detect the occupancy of AR within the regulatory regions of *DUSP4* (left) or *FOSB* (right). **h** KYSE410 cells were cultured in phenol red-free RPMI 1640 medium with 5% charcoal-stripped FBS. The cells were transiently transfected with siRNA targeting GATA3 or non-targeting siRNA for 48 h followed by R1881 treatment for 24 h. The expression of *DUSP4* (left) or *FOSB* (right) at the mRNA level was analyzed by qRT-PCR. **i** Enrichment of HDAC3 and SMRT ChIP-seq signals at R1881-induced AR binding sites in KYSE410 cells. **j–m** GATA3-depleted or control KYSE410 cells were treated with vehicle or R1881 (10 nM) for 4 h. Standard ChIP was performed to detect the occupancy of SMRT (**j, l**) or HDAC3 (**k, m**) on the regulatory regions of the *DUSP4* (**j, k**) or *FOSB* (**l**, **m**) gene locus. **n** KYSE410 cells were treated with DMSO, 10 μM enzalutamide, 25 μM enzalutamide, 10 μM HDAC3 inhibitor or a combination of enzalutamide and HDAC3 inhibitor for 96 h. The protein level of DUSP4 or FOSB was analyzed by western blotting. β-actin was used as loading control. **o** A total of 2000 KYSE410 cells were seeded into 6-well plates and then treated with DMSO, enzalutamide (25 μM), HDAC3i inhibitor (10 μM) or a combination of HDAC3 inhibitor (10 μM) and enzalutamide (25 μM) for 2 weeks. The number of colonies was counted and analyzed. **p** GATA3-de**p**leted or control KYSE410 cells were subcutaneously injected into male BALB/c nude mice. The mice in each group were randomly divided and treated with vehicle or enzalutamide (30 mg/kg) by oral gavage. The images of xenograft tumors are shown (left) and tumor weights in each group are quantified and analyzed (right). **q** Immunohistochemistry staining of ESCC tissues with AR and GATA3 antibodies. Representative images of two ESCC tumor sections were shown. Scale bar, 50 μm. **r** Correlation analysis between the expression of AR and GATA3 was performed ($n = 79$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

we found that GATA3 was the most enriched GATA family member in the AR binding regions of *DUSP4* and *FOSB* (Supplementary information, Fig. S6b–d). No significant change in GATA3 enrichment was observed between vehicle- and R1881-treated groups (Supplementary information, Fig. S6c, d), suggesting that GATA3 binds independently of the presence of androgen and thus may function as a pioneer factor. Moreover, we found that GATA3 forms a complex with liganded AR on chromatin (Supplementary information, Fig. S6e). Silencing of GATA3 significantly impaired R1881-induced AR binding to the *DUSP4* and *FOSB* loci (Fig. 1g; Supplementary information, Fig. S6f), attenuating repression of the *DUSP4* and *FOSB* genes (Fig. 1h; Supplementary information, Fig. S6g).

To reveal the molecular basis for GATA3-directed AR transcriptional repression, we sought to identify potential corepressors involved in this process. Our RNA-seq analysis revealed that NCoR, SMRT and their interacting enzyme HDAC3[9,10] were highly expressed in KYSE410 cells. All three corepressors exhibited a high basal level of *DUSP4* and *FOSB* occupancy in the absence of androgen, and the enrichment of SMRT and HDAC3 was significantly increased upon R1881 induction (Supplementary information, Fig. S7a–c). Importantly, the ability of R1881 to enhance recruitment of both SMRT and HDAC3 to androgen-induced AR binding sites was further validated at a genome-wide scale (Fig. 1i; Supplementary information, Fig. S7d–g). Given that GATA3 forms complexes with SMRT and HDAC3 on chromatin in the absence of hormone (Supplementary information, Fig. S8a, b), we next tested whether GATA3 is essential for the recruitment of SMRT and HDAC3 in the presence or absence of hormone. Silencing of GATA3 significantly decreased both basal and R1881-enhanced enrichment of SMRT and HDAC3 within the *DUSP4* and *FOSB* loci without affecting the protein levels of SMRT or HDAC3 (Fig. 1j–m; Supplementary information, Fig. S8c). Depletion of either SMRT or HDAC3 significantly increased the expression of both *DUSP4* and *FOSB* in KYSE410 cells at the RNA level (Supplementary information, Fig. S8d, e), and repressed KYSE410 and ZEC014 cell growth in vitro (Supplementary information, Fig. S8f–i). Importantly, pharmacological targeting of HDAC3 by the inhibitor RGFP966 (HDAC3i), counteracted R1881-induced repression of *DUSP4* and *FOSB* and significantly restored their expression (Supplementary information, Fig. S9a–c). Compared to either enzalutamide or HDAC3i alone, co-treatment with enzalutamide and HDAC3i caused a stronger increase in *DUSP4* and *FOSB* expression (Fig. 1n; Supplementary information, Fig. S9d, e) and a greater inhibitory effect on cell growth and/or survival (Fig. 1o; Supplementary information, Fig. S9f–i).

Consistent with the essential role of GATA3 in governing AR transcriptional repression in ESCC, we found that GATA3 silencing significantly decreased KYSE410 and ZEC014 cell growth and survival in vitro (Supplementary information, Figs. S6f, S10a–c), which can be rescued by additional silencing of *DUSP4* or *FOSB* expression (Supplementary information, Fig. S10d–f). Importantly, GATA3 depletion also inhibited ESCC growth in vivo (Fig. 1p). Interestingly, there were no significant differences in tumor weight between GATA3 silencing only and GATA3 silencing combined with enzalutamide treatment groups (Fig. 1p; Supplementary information, Fig. S10g). This indicated that targeting the AR upstream regulator GATA3 is sufficient to inhibit ESCC growth.

Finally, we examined GATA3-directed AR transcriptional regulation in ESCC patients. AR expression was positively correlated with GATA3 expression (Fig. 1q, r; Supplementary information, Fig. S11a–c) and negatively associated with expression of DUSP4 and FOSB (Supplementary information, Fig. S11d–f) in clinical ESCC samples. These data support the clinical relevance of GATA3/AR co-transcriptional repression of *DUSP4* and *FOSB*.

In summary, our findings reveal molecular mechanisms underlying AR transcriptional dysregulation in ESCC and identify the GATA3/AR/HDAC3 transcription axis as a promising target for future clinical trials treating male ESCC patients (Supplementary information, Fig. S12). Since the AR antagonist enzalutamide (XTANDI®) has already received FDA approval in the treatment of prostate cancer, a clinical trial evaluating the use of enzalutamide to treat AR-positive ESCC patients will be the next step towards an effective ESCC targeted therapy in the near future.

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AUTHOR CONTRIBUTIONS

F.H., H.C., Z.C., Q.W., Z.L. designed the study; F.H., H.C., X.Z., T.G., X.L., W.H., H.W., Z.C. performed the experiments; F.H., Z.C., Q.W., Z.L. analyzed the data; F.H., H.C., Z.C., Q.W., Z.L. wrote the manuscript.

ADDITIONAL INFORMATION

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Competing interests: The authors declare no competing interests.

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