ASTE1 frameshift mutation triggers the immune response in Epstein-Barr virus-associated gastric cancer

We also obtained RNA-seq data for 5 EBVaGC specimens from Kim's study\(^4\) and evaluated the PD-L1 expression-related gene panel (Supplementary Fig. S2d). Interestingly, these patients presented an immune-active phenotype, with comparable PD-L1 expression and a similar gene expression pattern to our immune-active patients. This finding explained the result of 100% ORR for PD-1 inhibitors in these five patients. Furthermore, two patients in our study with obvious progression were evaluated after three courses of treatment with nivolumab, a PD-1 inhibitor (Supplementary Fig. S2e), and both patients exhibited immune-inactive behavior.

We further built a gene mutation panel including ASTE1, ARID1A, TNFAIP6, SMAD4A, and GIPC1 to predict the immune state (Supplementary Fig. S3a), and mutations in this panel predicted an immune-active phenotype (sensitivity = 80.8%, specificity = 65.2%, AUC = 0.73, kappa = 0.463) (Supplementary Figs. S3b, S3c). Notably, mutations in ASTE1 were identified in six patients (12%), whose immune states were all active. The frameshift mutation R632Gfs*33 was identified in 5 of 6 cases with mutation (Fig. 1c). Interestingly, a better prognosis was observed in the ASTE1 mutant group\(^9\) (Supplementary Figs. S3d, S3e).

As revealed by gene set enrichment analysis (GSEA) comparing ASTE1-Mut with ASTE1-WT, "IFN-γ response" was the set most significantly enriched among upregulated genes (Fig. 1d). The expression levels of IFNG and CD274 were upregulated in immune-active cancer and in Wang's study\(^6\) (p < 0.001) (Supplementary Figs. S4a, S4b). Interestingly, mRNA expression of CD274 correlated positively with that of IFNG (r = 0.70, p < 0.0001) (Fig. 1e). Higher expression of PD-L1 was also validated at the protein level by immunohistochemistry (Supplementary Fig. S4c). Taken together, ASTE1-mutant cancer presents an active immune response, with a higher content of IFN-γ in the cancer microenvironment.

To explore the mechanism by which ASTE1 mutation activates the immune response, we knocked out the ASTE1 gene in the EV-ag cell line and selected KO#1 for transfection with ASTE1-MU (KO#1 + ASTE1\^{mut}), -WT (KO#1 + ASTE1\^{WT}) and the empty vector (KO#1 + EV) (Supplementary Fig. S5a). However, we did not observe any difference in cell proliferation or migration (Supplementary Figs. S5b, S5c). Interestingly, compared with the KO#CON group and KO#1 + ASTE1\^{WT} group, cell growth decreased significantly when cocultured with activated CD8\(^+\) T cells (Fig. 1f and Supplementary Fig. S5d). Moreover, we detected higher levels of IFN-γ in the coculture supernatant of the KO#1 + ASTE1\^{mut} group and KO#1 + EV group (Fig. 1g). In contrast, there was no change in the cell proliferation or immune response of CD8\(^+\) T cells when we overexpressed wild-type ASTE1 among EBV\(^+\) AGS cells (Supplementary Figs. S5e, S5f). To validate the observed PD-L1 upregulation induced by IFN-γ, we compared PD-L1 protein expression in different genotypes. Notably, the KO#1 + ASTE1\^{mut}
group and KO#1+EV group showed higher levels of PD-L1 protein after coculture or exogenous IFN-γ treatment (Fig. 1h).

Furthermore, ASTE1-Mut cancer presented higher expression levels of chemokines CXCL9, −10, and −11, ligands of CXCR3 (Fig. 1i, and Supplementary Figs. S6a, S6b). Also, expression of IFNG correlated with these chemokines ($r = 0.833$, 0.781, and 0.724 for CXCL9, −10 and −11, respectively, $p < 0.001$) (Fig. 1j and Supplementary Fig. S6c). In addition, qRT-PCR was performed to validate the increased CXCL9 and CXCL11 mRNA expression detected in the KO#1+ASTE1Mut and KO#1+EV cell lines (Fig. 1k...
Immune classification and immune activation triggered by ASTE1 mutation in EBVaGC. a Gene set variation analysis (GSVA) based on immune cell-related gene set. Differential expressed genes are closely related to immune cells. b Expression characteristics of immune subtypes according to functional gene panel. Cluster 2 upregulated most of the immune genes and was named “immune-active” type; cluster 1 was named “immune-inactive” type. c Lollipop plots of mutant ASTE1 in sequenced EBVaGC samples. Somatic mutations in ASTE1 were indicated at the top of the corresponding domains in the protein. Among a total of six identified mutations, five had truncating mutations at the same site (R632Gfs*33). d GSEA revealed an enrichment of “IFN-γ response” pathway in ASTE1 mutation group. Normalized enrichment score (NES) = 1.79. Nominal p-value = 0.03. FDR q-value = 0.17. e Expression of CD274/PD-L1 was associated with IFNG (r = 0.70, p < 0.0001). f Cell proliferation was measured using the CCK-8 assay per co-culture day. g IFN-γ in the supernatant of the co-culture system was measured by ELISA. h PD-L1 expression of carcinoma cells after co-cultured with CD8+ T cell or treated with IFN-γ i. ASTE1-mutant tumor upregulates CXCR3 ligand, CXCL9 (p < 0.01). The result was derived from RNA-seq of patient samples. j Expression of IFNG was associated with CXCL9 (r = 0.833, p < 0.001). k mRNA expression of CXCL9 in carcinoma cells was assessed by qPCR. l Left: results from western blot analyses of nuclear extracts and cytoplasm of transfected cells. Right: western blot analysis using anti-bodies against the respective phospho-sites (p) or total protein in NF-κB pathway. m mRNA expression of CXCL9 was detected by qPCR after RNAi knockdown. Expression of CXCL9 reduced significantly in KO#1 + EV group and KO#1 + MU group.

and Supplementary Fig. 56d). However, there was no difference between the expression of IRF 1 and CXCL10 or STAT1 and STAT3 (Supplementary Fig. 57). We hypothesized that effectors regulating the transcriptional inflammatory response program were involved, and we thus assessed p-p65 by immunofluorescence (Supplementary Fig. 58a) and observed it mainly in, but not limited to, the nucleus in EBV+ AGS cells (Fig. 11). ASTE1-WT decreased p-p65 expression, while ASTE1-Mut had little effect. It strengthened the point that wild-type ASTE1 may suppress the inflammatory pathway. In addition, the expression level of p-p65 was relatively less in cytoplasm but more in the nuclear, indicating ASTE1-Mut might promote its nuclear translocation. Notably, expression of CXCL9 was significantly decreased in the KO#1 + EV group and KO#1 + MU group after RelA/p65 Shh2 transfection (Fig. 1m and Supplementary Figs. 58b, 58c). These results indicated that ASTE1 mutation functions through activation of the NF-κB pathway in EBV+ GC cells.

ASTE1 mutation is reported to be associated with lymphocyte infiltration in MSI colorectal cancer. Here, we described for the first time the frameshift hotspot mutation R632Gfs*33 in ASTE1 (5/6) as being involved in EBVaGC. Strikingly, all EBVaGCs with ASTE1 mutations in our study were the immune-active type, which has been confirmed to predict an anti-PD1 response. Furthermore, ASTE1-mutant cancers show CXCL9-CXCR3 axis activation, which correlates with the efficacy of immunotherapy. Therefore, we propose a new mechanism by which ASTE1-mutant cancer cells autonomously express CXCL9 through NF-κB pathway activation, increase IFN-γ in the microenvironment and stimulate the immune response (Supplementary Fig. 59). Collectively, these findings strengthen the hypothesis that ASTE1 mutation has predictive potential for immunotherapy.

**ADDITIONAL INFORMATION**

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