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Activation of GATA binding protein 6 (GATA6) sustains oncogenic lineage-survival in esophageal adenocarcinoma

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Gene amplification is a tumor-specific event during malignant transformation. Recent studies have proposed a lineage-dependency (addiction) model of human cancer whereby amplification of certain lineage transcription factors predisposes a survival mechanism in tumor cells. These tumor cells are derived from tissues where the lineage factors play essential developmental and maintenance roles. Here, we show that recurrent amplification at 18q11.2 occurs in 21% of esophageal adenocarcinomas (EAC). Utilization of an integrative genomic strategy reveals a single gene, the embryonic endoderm transcription factor GATA6, as the selected target of the amplification. Overexpression of GATA6 is found in EACs that contain gene amplification. We find that EAC patients whose tumors carry GATA6 amplification have a poorer survival. We show that ectopic expression of GATA6, together with FGFR2 isomorph IIIb, increases anchorage-independent growth in immortalized Barrett’s esophageal cells. Conversely, siRNA-mediated silencing of GATA6 significantly reduces both cell proliferation and anchorage-independent growth in EAC cells. We further demonstrate that induction of apoptotic/anoikis pathways is triggered upon silencing of GATA6 in EAC cells but not in esophageal squamous cells. We show that activation of p38α signaling and up-regulation of TNF-related apoptosis-inducing ligand are detected in apoptotic EAC cells upon GATA6 deprivation. We conclude that selective gene amplification of GATA6 during EAC development sustains oncogenic lineage-survival of esophageal adenocarcinoma.

Cancer development is a multistage process involving both activation of oncopgenes and inactivation of tumor suppressor genes (9). Gene amplification is one mechanism for the activation of oncopgenes, and this activation can be causative for tumorigenesis (10, 11). Despite the complexity of genetic, epigenetic, and chromosomal abnormalities in a given cancer, inactivation of a single or a few initiating oncopgenes may impair tumor growth and survival, a phenotype termed “oncogene addiction” (12, 13). Recent studies of genomic amplification in cancer demonstrated that certain master regulatory factors, involved in both embryogenesis and subsequent tissue maintenance, are often selectively amplified in tumors arising from the lineages where the factors play an important developmental role (14–17). In the present study, we identify a highly amplified transcription factor, GATA6, in EAC using integrative genomic approaches and show that GATA6 has properties of a lineage-survival oncopgene in EAC.

Results

Integrative Genomic Analysis in EAC Identifies Recurrent Amplification at 18q11.2 and a Single Selected Target Gene, GATA6. We performed array-based comparative genomic hybridization (array-CGH) in 20 EAC samples and identified genomic amplification at 18q11.2 as a recurrently amplified locus (Fig. 1A). Three of 20 EAC samples assayed by array-CGH showed amplification with one tumor (T8) containing an amplified unit about 706 kb that included only two genes, GATA6 and CTAGE1 (Fig. L4, and SI Appendix, Fig. S1A and Table S1A). We next analyzed DNA dosage of five genes spanning a 1.5-Mb segment of the 18q11.2 region, including both GATA6 and CTAGE1, in a cohort of 85 EACs using real-time PCR (qPCR) (Fig. 1B and SI Appendix, Fig. S2). The 2-ΔΔCT calculation of cycle threshold was performed (18). The cutoff value was arbitrarily set at ≥1.9, which represented greater than 4N of the haploid genome, given ≥70% tumor cell content of the EAC samples studied. GATA6 amplification was found in 18 of 85 EACs (21.2%), which is significantly higher than the other four genes coamplified in the ampiclon (P < 0.001) (Fig. 1C, and SI Appendix, Fig. S1C and Table S1C). The highest DNA copy number (>36N) was also found in the GATA6 gene.

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(Fig. 1 B and C). We further validated the 18q11.2 amplification in 73 of the 85 EAC samples using genome-wide 250 K Sty I SNP arrays. Consistent with array-CGH and qPCR results, the 18q11.2 amplicon was found to be a confined chromosomal segment with the core amplified-domain about 93 kb and including only GATA6 (Fig. 1 D and E, and SI Appendix, Fig. S1B and Table S1B). GATA6 was found to be amplified in 15 of 73 EACs examined (20.5%), with the cutoff value of log2 ratio ≥ 0.848 (16). The results of GATA6 amplification in these samples assayed both by SNP array and qPCR were highly correlated (r = 0.92, P < 0.0001). Kaplan–Meier survival analysis in 97 EAC samples indicated that patients with the GATA6 amplicon had a poorer survival (P = 0.0292) (Fig. 1F) and the amplification was not related to tumor stage (χ2 = 2.962, P = 0.0853) (SI Appendix, Table S3). In addition, we did not find any consistent deletion at the GATA6 locus in 73 EAC SNP arrays (Fig. 1 D and E). We further examined eight additional SNP markers dispersed in the GATA6 gene region and sequenced the full-length GATA6 coding region in 22 EACs (SI Appendix, Table S4). We did not find any mutations in the GATA6 coding sequence or deletions at the GATA6 locus.

**Gene Amplification Drives the Overexpression of GATA6 in EACs.** Transcriptional expression of GATA6 among 50 EACs, including all amplified tumor specimens available, was assessed using quantitative RT-PCR (qRT-PCR) (Fig. 2A and SI Appendix, Fig. S2C). The value of 2ΔΔCt ≥ 2 (twofold) relative to normal intestinal RNA was set as the threshold for gene overexpression. Fourteen of these 30 EAC samples contained GATA6 amplification; among them, 13 (93%) were found to overexpress GATA6 (r = 0.850, P < 0.0001). Only 9 of 30 EACs were found to have MIB1 amplification (SI Appendix, Fig. S1C), and five of these nine samples overexpressed MIB1 (r = 0.875, P = 0.8406) (Fig. 2A and B). The change in GATA6 expression in tumors containing GATA6 amplification was significantly greater than that in tumors without GATA6 amplification (P < 0.001) or in tumors with or without MIB1 amplification (P < 0.001) (Fig. 2B). Overexpression of GATA6 protein in these cases was confirmed using Western blot and immunohistochemistry with an esophageal tissue microarray (TMA) (Fig. 2 C and D). Ten of 13 GATA6-amplified EAC TMA cores demonstrated strong staining, whereas only seven were found to contain MIB1 amplification and four of the seven MIB1-amplified EACs had positive MIB1 staining (Fig. 2D). Furthermore, when we analyzed a multicancer study of gene-expression profiling using the Oncomine database (www.oncomine.com), we found that GATA6 was one of the signature genes with high expression that distinguishes gastrointestinal carcinomas from other tumor types (SI Appendix, Fig. S3).

**Ectopic Expression of GATA6 Increases Anchorage-Independent Growth in Immortalized Barrett’s Cells in Collaboration with FGFR2IIib.** Given that GATA6 is an embryonic gut lineage transcription factor and that gene amplification of GATA6 is selected during development of EAC, we hypothesized that GATA6 exerts an oncogenic lineage-survival role in EAC. We found that GATA6 alone was not transforming, as determined by anchorage-independent assays using the IGV software. The y axis shows a descending log2 copy number ratio in 73 EACs. Horizontal bars represent individual tumor samples. The boxed area shows a 4-Mb region in the vicinity of the 18q11.2 amplicon with the arrow indicating the location of GATA6. (E) Magnified view of the 4-Mb region of the 18q11.2 amplicon from D. Boxed region with yellow lines shows the smallest amplified unit defined in 73 EACs. (F) Kaplan–Meier survival plots estimate a poorer clinical outcome (P = 0.0292) in EAC patients bearing the 18q11.2 amplicon in their tumors.

![Fig. 1](image-url)
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tumor volume and weight, one of six FloA/pBMN6 xenografts
Xenograft tumor growth was not observed when 2 × 106 CP-A or

dogenous GATA6 were transduced with GATA6 pBMN6 (SI Appendix, Fig. S5B), a consistent increase in cell proliferation was not observed (SI Appendix, SI Note, and Fig. S6 A–C). However, ectopic expression of GATA6 in cells significantly increased DNA synthesis and S-phase cell cycle distribution in BrdU incorporation assays (SI Appendix, Fig. S6 D and E). A subsequent reduction of S-phase fraction at 48 h following GATA6 transduction was also observed and we speculated that a temporal onset of inducible p21/INK4A (21) might account for this observation (SI Appendix, Fig. S6 F and G), indicating the complexity of GATA6 function in these cells.

We further investigated GATA6 modulated tumorigenicity and tumor growth in immune compromised NOD SCID-γ (NSG) mice. Immortalized Barrett’s CP-A cells or EAC FloA cells (FloA cells are derived from Flo-1 cells through soft agar selection) were transiently transduced with expression constructs either pBMN-GATA6(pBMN6) or pBMN-LacZ (pBMN-Z) and subjected to subcutaneous implantation (SI Appendix, Fig. S7A). Xenograft tumor growth was not observed when 2 × 106 CP-A or CP-A/FGFR2IIIb cells with pBMN6 or pBMN-Z were injected in NSG mice for 7 wk. Although most of the tumors that formed at 7 wk in NSG mice after injection with either 5 × 106 of FloA, pBMN-Z or FloA/pBMN6 xenografts grew aggressively with significantly large tumors that invaded through adjacent muscle tissue to the abdomen and the bone of the hind leg (SI Appendix, Fig. S7 A–E).

GATA6 Sustains EAC Cell Growth and Survival in a Lineage-Specific Manner. We next analyzed the growths- and survival-dependency of GATA6 in EAC. Barrett’s and esophageal squamous cell lines that endogenously or exogenously express GATA6 (SI Appendix, Fig. S5B and Table S6) using siRNA-mediated knockdown assays. Cell proliferation in both Barrett’s cells (CP-A and CP-B) and EAC cells (OE33 and Flo-1 with stably transduced GATA6)

Fig. 2. Overexpression of GATA6 driven by gene amplification in EACs. (A) qRT-PCR analysis of 30 EACs. The y axis shows fold-changes (2−ΔΔCt) of gene expression relative to the normal intestinal tissue (IntN) as GATA6 expression was found to be extremely low or absent in esophageal squamous epithelia (e.g., 43N, and N27 in Q). Overexpression of GATA6 was detected in 13 of 14 EACs containing the GATA6 amplicon. GATA6 up-regulation was also observed in a subset of dysplastic Barrett’s samples (e.g., 19B). All qRT-PCR reactions were repeated three times. (B) Boxplot analysis of the qRT-PCR data. The y axis represents fold-changes in gene expression relative to the expression of normal intestinal RNA (***P < 0.001, Student’s t test). (C) Western blot analysis. Only a small set of primary tissues were examined because of sample availability. Overexpression of GATA6 is shown in a GATA6-amplified EAC (T27) but not in EACs without the GATA6 amplicon (T34 and T78). Samples T27 (EAC), G27 (normal gastric), and N27 (normal esophageal squamous mucosa) were derived from the same patient. (D) Immunohistochemistry of GATA6 and MIB1 in EAC TMAs. Overexpression of GATA6 protein was detected in EACs with amplified GATA6 (representative T27, T70, and T83) compared with EAC without GATA6 amplification (T9) (Magnification ×10). MIB1 expression was only observed in tumor T83 that contains MIB1 amplification (Magnification ×20).

construct pBMN6 (Fig. 3B). We found that GATA6 conferred significantly enhanced anchorage-independent growth in CP-A cells in the presence of FGFR2IIIB (Fig. 3), although the mechanism of interdependency for cellular transformation between GATA6 and FGFR2IIIB is yet to be determined.

There are only a few EAC cell lines available worldwide. The cell lines used in this study, including Flo-1 and OE33 (SI Appendix, Table S5) EAC lines, CP-A and CP-B immortalized Barrett’s cells (20), and Het-A1 and TE13 esophageal squamous cell lines, expressed different levels of GATA6 and none have GATA6 amplification, except for TE13, which has 4–5N copy numbers of GATA6 relative to Flo-1 cells (SI Appendix, Fig. S5 and Table S6). We investigated whether a high level of ectopic expression of GATA6 would alter cell proliferation in cultured EAC cells. When Flo-1 cells that expressed very limited en-
was decreased following knockdown of GATA6, with more significant reduction observed in EAC cells than in Barrett’s cells (Fig. 4A and B). In contrast, this reduction of cell proliferation upon knockdown of GATA6 was not found in esophageal squamous cells Het-1A and TE13 (SI Appendix, Fig. S8). We further observed that siRNA-mediated silencing of GATA6 significantly reduced anchorage-independent growth in OE33 cells (Fig. 4C). Morphological changes characteristic of cell death were observed in EAC cells but not in squamous cells (Fig. 4D). GATA6 silencing induced significant DNA fragmentation indicative of cellular apoptosis in EAC cells (OE33) but not in non-EAC lines (TE15 and Het-1A), as determined by BrdU/TUNEL assays (Fig. 4E–G). To further determine the apoptotic phenotypes induced upon silencing of GATA6, we assessed anoikis, a specific type of apoptosis (22), in Flo-1/GATA6 stable cells. We found that silencing of GATA6 enhanced anoikis as determined by increased poly(ADP-ribose) polymerase (PARP) cleavage, an indicator of caspase 3 activation (Fig. 5A). To validate the effects of siRNA-mediated silencing of GATA6, we performed assays using two additional siRNAs that targeted different coding sequences of GATA6 (SI Appendix, Table S7). PARP cleavage was observed in all three siRNA-treated EAC OE33 cells, but not in squamous TE13 cells (Fig. 5B). In addition, all three siRNA fragments targeting GATA6 significantly increased caspase 3 activity in OE33 cells compared with TE13 cells (Fig. 5C) and produced similar apoptotic phenotypes following GATA6 knockdown (Fig. 5D and SI Appendix, Fig. S9A). Changes in cellular senescence were not found in non-GATA6-expressing EAC Flo-1 cells upon ectopic expression of GATA6 or in siGATA6-transfected OE33 cells using senescence-associated β-galactosidase staining (SI Appendix, Fig. S9B).

Activation of p38α Signaling and Up-Regulation of TNF-Related Apoptosis-Inducing Ligand upon siRNA-Mediated GATA6 Withdrawal in EAC Cells. Because GATA6 is a spatial and temporal master regulator in embryonic development (23) and its deprivation causes massive apoptosis in embryonic ectoderm (24), we speculate that gene amplification-induced differential expression of GATA6 in EAC may cause transcriptional reprogramming in tumor genomes. We used two model EAC cell lines, endogenously-limited GATA6-expressing Flo-1 cells and GATA6-expressing OE33 cells (SI Appendix, Fig. S5B). For transcriptional profiling using Affymetrix U133A arrays. Cell RNA was harvested at 24 h to assess an acute response and at 72 h for analysis of the sustained effect of GATA6 regulation following transient transduction of GATA6 (>900-fold) in Flo-1 cells or siRNA-mediated knockdown of GATA6 (>90%) in OE33 cells (SI Appendix, Fig. S10A). Analysis of array data revealed that many genes in diverse cellular pathways were transcriptionally reprogrammed upon differential expression of GATA6 (SI Appendix, Fig. S10B). We were particularly interested in the genes relative to apoptotic pathways following GATA6 silencing in OE33 cells (SI Appendix, Table S8). The proapoptotic genes, TNFSF10/TNF-related apoptosis-inducing ligand (TRAIL), XAF1, and DAPK2, were among the top 100 genes up-regulated upon GATA6-silencing (Fig. 6A and SI Appendix, Table S8), and the results were validated in an
independent experiment with siGATA6-treated OE33 cells using real-time RT-PCR (Fig. 6B). TNFSF10/TRAIL is a death ligand of the TNF family and has been shown to preferentially induce apoptosis in transformed tumor cells (25). TRAIL protein was up-regulated whereas the prosurvival protein BCL-2 was down-regulated following 60-h treatment with siGATA6 in apoptotic OE33 cells (Fig. 6C). The fact that cleaved caspase 3 but not caspase 9 was increased (Fig. 6D) in apoptotic OE33 cells treated with siGATA6 indicates that siGATA6-induced apoptosis in EAC cells may be through the extrinsic apoptosis pathway. Using a phospho-kinase array, we confirmed that the differential expression of GATA6 in FloA or OE33 EAC cells led to modulations of many diverse kinase signaling pathways, including the three MAPK pathways, MEK1/2/ERK1/2, JNK, and p38α (Fig. 6E). In particular, we observed that stress-activated protein kinase p38α/MAPK14, which can induce apoptosis through several mechanisms, including activation of proapoptotic proteins and activation of prosurvival signals (26), was down-regulated in GATA6-transduced FloA cells and up-regulated in siGATA6-silenced OE33 cells (Fig. 6E), and the results were further validated by Western blot analyses (Fig. 6F).

Discussion

GATA6 is a member of the highly conserved GATA family, which is composed of six zinc-finger transcription factors that regulate lineage-restricted development, differentiation, and cellular aging (27–29). GATA1-3 are essential for formation and differentiation of pluripotent and multipotent hematopoietic stem cells (30), whereas GATA4-6 are indispensable for the lineage-specific development and differentiation of cells of endodermal and mesodermal origin (24, 31). Inactivation of GATA6 in the mouse embryo causes embryonic lethality (24, 32). GATA6 is thought to be a master regulator because inactivation of GATA6 resulted in loss of expression of all hepatocyte nuclear factors in knockout mice (23, 33). In the adult gastrointestinal tract, GATA6 is more localized and expressed within the proliferative and lineage stem-cell zone at the bottom of the gut crypts (34, 35).

Consistent with the idea that GATA6 amplification is a lineage-specific activation, GATA6 amplification was not observed in esophageal squamous carcinoma, as reported in our recent study (16). Interestingly, Kwei et al. (36) and Fu et al. (37) reported that 18q11.2 gain/amplification with overexpression of GATA6 is detected in 9–19% of pancreatic carcinomas. Both the pancreas and distal esophagus are derived from the embryonic endodermal foregut, making it plausible that these two tumors may share common lineage-survival oncogene. Although GATA6 has been reported to be a tumor suppressor in glioma (38) and ovarian (39) cancers, which are tissues of nonendodermal origin, recent comprehensive studies have failed to uncover any evidence for genomic alterations of GATA6 in these diseases (40, 41).

Amplification of lineage-survival oncogenes imposes survival mechanisms in tumor cells. These factors are otherwise involved in lineage precursor cell development and differentiation (14–17). We hypothesized that GATA6 is a lineage-survival oncogene in EAC based on the fact that GATA6 is a master regulator and stem cell-lineage transcription factor in embryogenesis and that GATA6 amplification is a selective event during the development and progression of EAC. We demonstrated that siRNA-mediated silencing of GATA6 decreased both cell proliferation and anchorage-independent growth in EAC cells and caused a variety of apoptotic phenotypes. The fact that direct tumorigenicity was not confirmed in immortalized Barrett’s CP-A cells indicates that amplification-led overexpression of GATA6 in EAC may impose survival and “stemness” to the esophageal cells under chronic attack from gastro-esophageal reflux and subsequent inflammatory environment, rather than play a role in EAC initiation or formation. We observed that modifying the expression of GATA6 in EAC cells induced broad cellular responses. Specifically, we demonstrated that differential expression of GATA6 caused changes in p38α activation, as well as modulation in the TRAIL-mediated apoptotic pathway. Clearly, further experiments are required to fully understand the oncogenic lineage-survival role of GATA6 in cellular transformation and progression.
of esophageal adenocarcinoma. In light of the lineage-addiction model of human cancer, our present study suggests that thera-
pic deprivation of GATA6 in GATA6-amplified EAC patients may improve patient survival.

**Materials and Methods**

**Patients and EAC Samples.** All animal studies were conducted under the guidelines and approved protocols from the University Committee on Use and Care of Animal of the University of Michigan. Written consent was obtained from each patient according to the approval and guidelines of the University of Michigan institutional review board. Tissues were obtained from patients undergoing esophagectomy for adenocarcinoma at the University of Michigan Health System between 1991 and 2004. Patients in this study had no preoperative radiation or chemotherapy. Specimens were fresh-frozen in liquid nitrogen and stored at −80 °C until use. Cellularity of metastatic, dysplastic, and tumor samples were assured to be greater than 70% before sample DNA, RNA, or protein was isolated. DNA, RNA, and protein isolation procedures are in **SI Appendix, SI Materials and Methods.**

**Tiling Path Array-CGH and Data Analysis.** DNA copy number profiles were generated for 20 EACs using a whole-genome tiling path array, as previously described (42). Data analysis details are in **SI Appendix, SI Materials and Methods.**

**SNP Array Experiments and Analysis.** SNP arrays were performed as previously described (16). Briefly, 73 EAC DNAs were genotyped using the Genome-Wide Human Sty 1250K SNP Array (Affymetrix). Copy number analyses with SNP arrays were performed as a log2-copy number ratio exceeding 0.848 for amplifications and −0.737 for deletions. Genomic positions were mapped in the hg18 genome build. SNP data were visualized using the software IGV 1.3.1 (Integrative Genomics Viewer, www.broadinstitute.org/igv).

**Immunohistochemistry of TMA.** Briefly, TMA arrays contained 122 sections from 73 EAC patients, including 63 EAC sections, 18 mixed sections of EAC and dysplasia, 22 Barrett’s metaplastic and dysplastic sections, 9 metastatic lymph nodes, and 10 normal sections of various tissue types. Procedure details are in **SI Appendix, SI Materials and Methods.**

**Cell Lines and Culture Conditions.** CP-A and CP-B cell lines were kind gifts from Peter Rabinovitch (University of Washington, Seattle, WA). CP-A and CP-B were derived from Barrett’s metaplasia and high-grade dysplasia, respectively, and were immortalized through induction of hTERT (20). Procedure details are in **SI Appendix, SI Materials and Methods.**

**Statistical Analysis.** Kaplan-Meier survival was computed using the GraphPad Prism software and P values were determined by a log-rank test. Box plot analyses were determined using SigmaPlot software. Analyses in t-test, one-way ANOVA, and correlation coefficient were applied for all necessary experiments.

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