Rearrangement processes and structural variations show evidence of selection in oesophageal adenocarcinomas

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Oesophageal adenocarcinoma (OAC) provides an ideal case study to characterize large-scale rearrangements. Using whole genome short-read sequencing of 383 cases, for which 214 had matched whole transcriptomes, we observed structural variations (SV) with a predominance of deletions, tandem duplications and inter-chromosome junctions that could be identified as LINE-1 mobile element (ME) insertions. Complex clusters of rearrangements resembling breakage-fusion-bridge cycles or extrachromosomal circular DNA accounted for 22% of complex SVs affecting known oncogenes. Counting SV events affecting known driver genes substantially increased the recurrence rates of these drivers. After excluding fragile sites, we identified 51 candidate new drivers in genomic regions disrupted by SVs, including ETV5, KAT6B and CLTC. RUNX1 was the most recurrently altered gene (24%), with many deletions inactivating the RUNT domain but preserved the reading frame, suggesting an altered protein product. These findings underscore the importance of identification of SV events in OAC with implications for targeted therapies.
Patterns of rearrangement can reflect the underlying mechanism generating the rearrangement, genetic instabilities or mutagen exposures, and these may in turn determine response to therapy or help explain the underlying aetiology1,2. Rearrangements in driver genes, such as deletions, amplifications, gene breakages and gene fusions, seem likely to be at least as important a source of driver mutations as single nucleotide variants (SNVs) and indels in many carcinomas3–5. The Pan-Cancer Analysis of Whole Genomes (PCAWG) analysed whole genome sequencing data from multiple cancer types and this revealed a remarkable heterogeneity of SVs. In some cancer types, such as breast and ovary, it was estimated that up to three times more driver genes are altered by SVs than by SNVs and indels3. Nevertheless, our ability to identify SV driver events lags behind that of SNV and indel events. This is primarily because there is no measure of the background SV mutation rate, unlike synonymous SNV mutations, that enable the identification of driver genes disrupted by SVs and rearrangements often involve large genomic regions6,7.

Oesophageal cancer, especially the subtype oesophageal adenocarcinoma (OAC), emerged from the PCAWG analysis (n = 100 OACs) as a cancer type with one of the highest burdens of SVs with complex rearrangements1,9. These include breakage-fusion-bridge (BFB) cycles; catastrophic chromothripsis events with oscillating copy number patterns8, deletions in the fragile-sites and the highest rate of somatic mobile element (ME) inserts of any cancer type1,9. MEs are mainly inserts from Long Interspersed Nuclear Element-1 (LINE-1) retrotransposons, and can consist either of LINE-1 sequence alone or LINE-1 with up to a few kb of 3’ flanking unique genomic sequence transduced11,13. Driver alterations in SVs and indels are well characterized in OAC, as are distinct copy number (CN) amplification of oncogenes (e.g. ERBB2, EGFR, RB1, GATA4/6, CCND1 and MDM2) and loss of tumour suppressors (e.g. TP53, CDKN2A, CDKN2B)8,10,14. Rearrangement processes such as BFB cycles and extrachromosomal circular DNA (ecDNA) have been shown to result in copy number amplification in key oncogenes15–18 while a variety of SVs can disrupt tumour suppressor genes, including LINE-1 insertions11,14. However, to date the analysis of these complex events in OAC has not been performed at the detail required to fully elucidate the spectrum and underlying mechanisms for complex SVs.

In this analysis we combine recent advances in methods for dissecting complex rearrangements and identifying driver events13,11,19–22 to characterize SVs in a large cohort of 383 OACs with paired whole transcriptome sequence (WTS) in a subset (n = 214). Coupled with detailed clinical annotation, this analysis has enabled us to establish the functional relevance of the driver genes affected by these rearrangements.

**Results**

**Rearrangement patterns in OAC genomes show frequent mobile element insertions and complex SV.** We analysed 383 OAC genomes and observed a wide variation in the numbers of structural variants (SV) between cases, with a predominance of deletions (DEL), inter-chromosome junctions (BND) and tandem duplications (DUP) (Fig. 1a). The SV were deconvoluted into rearrangement signatures (RS, Supplementary Fig. S1A) by combining the types of SVs with the size and degree of clustering23, mapped to known signatures (Supplementary Fig. S1B)24 and clustered to distinct profilsoft arrangements in different groups of patients (Fig. 1b, c, Supplementary Fig. S2A, Supplementary Data 1). Six RS were identified: two with DEL sizes of 1–10 kb and 100 kb–1 Mb (signatures RS7 and RS9, respectively); a non-clustered inter-chromosomal junction (BND) (RS2) and a clustered inter-chromosomal junction signature (RS4); and a clustered SV signature with a high number of DELs, INVs, and DUPs of size 1–10 Mb, corresponding to a combination of signatures (RS6a and RS12) and a non-clustered 100 kb–1 Mb DUP signature RS13,14. We identified a lower burden of focal amplifications and extrachromosomal DNA (ecDNA) cycles in the RS7 + RS9 group (p = 0.0056, p = 0.0061, respectively, Wilcoxon rank sum test, Supplementary Fig S2B, C), an enrichment of mobile element (ME) insertions in the RS4 group (p = 4 × 10−11) and complex clusters of SVs in the RS1 group of patients (p = 8.2 × 10−7, Fig. 1d–g, Supplementary Fig. S2B–G).

To determine the contributions of ME insertions in generating SVs in OAC, we used the Trafic algorithm11,13, which identified a median of 60 (IQR 3–117) ME inserts per tumour (Fig. 1f, Supplementary Data 2). The majority of inserts (81%, 37,475) were of LINE-1 sequence alone (‘sole’), while 19% (8517) included transduced 3’ flanking sequence. Of these 7% (3195) retained LINE1 sequence, while 12% (5322) were ‘orphan’ transductions, i.e., transduced sequence alone (Fig. 1f, Supplementary Data 2). Since transduced sequence reveals the origin of the LINE-1 in the genome, we could assign 13% (6109) to germline elements and, remarkably, 5% (2408) to novel, somatically acquired elements. In the tumours with the highest numbers of inserts, the active germline LINE-1s were generally those described by Rodriguez-Martin et al. as ‘Plinian’, i.e., rarely present but with high activity when activated. This is in contrast to the ‘Strombolian’ germline LINE-1 elements, which are frequently active in cancer and tend to be active in tumours with fewer inserts3,11. We also identified ME insertions among our conventional SV calls and as most are inter-chromosomal, most resemble translocations. Hence, there were 13,189 inter-chromosomal junctions that had at least one breakpoint overlapping with a ME called by Trafic in the sample (Supplementary Data 2). Rearrangement signatures in OAC correspond to processes leading to ME insertions, DNA damage repair and complex rearrangements. To identify the features of biological processes associated with each RS, we carried out a logistic regression based on the presence of each RS in each tumour and orthogonal features including the number of ME insertions; chromothripsis events, complex SV clusters, SNV signatures subtypes10, BFB or ecDNA events numbers and in known driver genes (Supplementary Data 3).

RS4, a signature of unknown aetiology consisting of clustered inter-chromosomal junctions (affecting 74% of cases), was strongly associated with the number of ME insertion events (log odds: 6.13, p = 3.21 × 10−9, Supplementary Data 4). We further determined if each inter-chromosomal junction cluster overlapped with nearby ME insertions or source elements and found 59% (1622/2751) of RS4 clusters overlapped with ME insertions—41% called by Trafic, while the remaining 18% of RS4 clusters overlapped with regions with previous evidence of transductions by MEs11,13 (Supplementary Data 5). We also identified an association with the number of ecDNA amplicons (log-odds = 0.46, p = 0.009, Supplementary Data 4) and increased KRAS expression (log odds 0.54, p = 5.47 × 10−4, logistic regression, p = 0.026, Wilcoxon rank sum test) in tumours with RS4, driven by tumours (15/19) with both ME insertions and KRAS amplification (Supplementary Fig. S2H, Supplementary Data 6). In addition, RS4 was associated with a lower expression of Leucine Rich Repeat Kinase 2 (LRRK2), a gene with interactions with ATM and roles regulating MDM2 and TP53 in DNA repair pathways25 that was previously identified14 (log odds = −0.99, p = 4.91 × 10−4, Supplementary Data 4).
addition, we observed that tumours with RS4 had an increased frequency of SVs in genomic regions containing MDM2, H3F3B, PTPRB and GRM3 compared to tumours devoid of RS4 (Supplementary Fig. S2I). Signature RS2 (87%) was associated with a lower number of ecDNA amplicons involving ERBB2 (log odds $-0.89$, p = 0.033, FDR = 0.051, Supplementary Data 4). Tumours with a high proportion of SVs assigned to RS2 have a low burden of SV events ($p = 0.0181$, Wilcoxon rank sum test, Supplementary Fig. S2A) and are genomically stable compared to other tumours.

The deletion signature, RS7 (69%) was associated with an absence of ME insertions (log odds $-1.53$, p = 2.85 x 10^{-8}) and a higher burden of the SNV signature SBS17a (log odds $3.33 	imes 10^{-6}$). RS9 (68%) was associated with the presence of the DNA damage response (DDR) phenotype based on SNV signatures described by Secrèr et al. $^{10}$ (log odds $1.41$, p = $1.71 	imes 10^{-4}$) and a lower number of ecDNA cycles affecting the cell cycle regulator Cyclin E1 (CCNE1, log odds $-1.14$, p = 0.025, Supplementary Data 4).

Signature RS1 (47%) was associated with ecDNA events encompassing (log odds $3.28$, p-value = 0.004, logistic regression, Supplementary Data 4) and increased expression of CCNE1 compared to other tumours (log odds $0.88$, p-value = $1.55 	imes 10^{-4}$, logistic regression, Supplementary Data 4, p = $7.5 	imes 10^{-7}$, Wilcoxon rank sum test, Supplementary Fig. S2I). Tumours with RS1 were associated with an absence of ecDNA spanning CDK6 (log odds $-1.18$ p = 0.008) and low ME insertions ($-1.14$, p = $2.55 	imes 10^{-4}$, Supplementary Data 4). RS1 corresponded to the tandem duplication phenotype signature, associated with high CCNE1 expression ($p = 3.6 	imes 10^{-6}$, Wilcoxon rank sum test, Supplementary Fig. S2I) and replication stress, previously reported in breast, ovarian, stomach and liver cancer$^{2,26-28}$.

The 'clustered' signature RS6a + RS12 (69%) was associated with complex SV including a higher number of ecDNA and BFB cycles (log odds $0.47$, p = 0.003, log odds $0.69$, p = $9.19 	imes 10^{-5}$, respectively). Complex rearrangements consisting of clustered inversions and foldback inversions made up 20%
of SV clusters associated with RS6a + RS12, and many additional clusters containing larger complex events (Fig. 1e, g).

Five example tumours are shown, respectively, with a high proportion of predominant deletions (RS7 + RS9); non-clustered SVs (RS2); densely clustered SV inversions (RS6 + 12); inter-chromosomal junctions overlapping LINE-1 ME insertions (RS4) and clusters of tandem duplications (RS1) (Fig. 1h).

Complex SVs involving known oncopgenes in OAC can be explained by ecDNA ampiclons. Complex clusters of rearrangements are thus a prominent feature in OAC and we sought to identify clusters which are likely due to the formation of BFB cycles consisting of foldback inversions and circular ecDNA events that alter known oncogenes. We identified ecDNA events by applying the Amplicon Architect tool, that starts from regions estimated by CNVKit to have an absolute copy number (median CN = 12, IQR 7.9–19.1) and positive correlation (Pearson’s correlation = 0.42, p = 2.993 × 10⁻⁷) with high gene expression (Fig. 2b). In addition, several likely driver genes were co-amplified in large complex ampiclons, notably CCR7, and/or RARα-amplified with ERBB2 (Fig. 2c, Supplementary Fig. S3); and AKA9 and/or GATA1 with CDK6 (Supplementary Fig. S3).

It is instructive to consider individual cases. For example, in a tumour with 28 SV breakpoints in two clusters around the highly amplified CDK12-ERBB2 (copy number = 115) and STAT5B-STAT3 (copy number = 72) loci, reconstruction suggested there were multiple ecDNA circles or segments carrying either ERBB2 or STAT5B alone, plus some carrying both ampiclons (copy number = 25). The combined structure was consistent with a circular ecDNA structure (Fig. 2c, d) that included two clusters of enhancers (hg19 chr17:37773759-37939651, chr17:39768677-39852129). The enhancers were identified in publicly available OAC tumours and OAC cell lines data and the ecDNA encoded a CDK12-STAT5B fusion, that was confirmed using RNA sequencing. Similarly, an EGRF-SEC61G fusion previously predicted from DNA sequencing in a PCAWG study, proved to be in an EGRF amplification that was part of a cycllical ecDNA with enhancer marks on both chromosome segments (chr13:33846776-33860433, chr7:55132499-55154521, Fig. 2c, f).

Identifying SVs in OAC driver events. To assess the contribution of SVs to driver events we first considered genes that we had previously identified to be targets of SNV, indel, amplification and deletion driver events. We identified likely additional driver events due to SVs where the interval between two breakpoints overlapped an exon or exons of known driver gene. Adding these SV events substantially increased the recurrence rates of known drivers. For example, among major tumour suppressors, recurrence CDKN2A increased from 25% to 43% and SMAD4 from 14% to 27%, PTEN from 4% to 17% and APC to from 10% to 22% while TP53 showed a predominance of SNV alterations (Fig. 3, Supplementary Data 9).

We carried out a two-proportions-z-test to compare the recurrence of all 48 canonical drivers (p-value = 2.2 × 10⁻¹⁶) and in each individual gene, with and without considering SVs. Aside from four genes (TP53, AXIN1, NOTCH1, STK11) known to be affected by SNVs, 44 out of 48 genes show a significantly higher recurrence when SVs are considered (Supplementary Data 9).

Next, we attempted to identify OAC driver genes affected by SVs, or “hotspots”, characterized by more frequent breaks per unit of genome (1 Mb bins, 500 kb overlapping), after removing known fragile sites, and regions flanking ampiclons and deletions. By comparing the recurrence and density of SVs in each hotspot, we identified that fragile sites and copy number altered hotspots obscured driver genes affected by SVs and selected a method that adjusts for CN alterations and other genomic context (Fig. 4a). We identified hotspots in two steps, the first using a previously published method that accounts for genomic context. Secondly to find focal SVs, we used a consensus approach where bins had to be identified in at least two of the following methods: (1) background distribution modelling of SVs in a whole-genome, (2) per-chromosome context and (3) rank-sum-k-means clustering (see the “Methods” section). We further required that the genes to be listed as cancer-relevant by the CGC/COSMIC database.

A total of 108 regions (1 Mb bins, or groups of adjacent bins) with frequent breaks were identified in either the genomic-context dependent model or focal approach and 41 regions contained known COSMIC genes (Fig. 4b, Supplementary Data 10). These included bins containing RUNX1, MALAT1, RAD51B, COX6C, GPHN, NBN, KAT6B, CLTC, ETV5 and PTPRD that were identified by both approaches (Fig. 4b, c, Supplementary Data 10, 11). We noted that PTPRD and GPHN were identified as genes in possible fragile sites and excluded them from further analyses. As the COSMIC genes present in hotspots might not be directly affected by SVs, we narrowed down driver gene candidates using the criteria of the SV spanning or overlapping the gene by intersecting the genomic region between the start and end position for intra-chromosomal SVs and between the start and end of both breakpoints in an inter- chromosomal breakend (defined by MANTA) with exons of a gene. Sixty-one candidate genes were identified with RUNX1 as the most recurrent deleted as many of the SVs in the regions overlapped the gene (Fig. 4b, c), and this is discussed in detail below.

Aside from RUNX1, CDKN2A, BCL3 and MYB were identified, with predominant focal deletions affecting CDKN2A and duplications affecting BCL3 and MYB (Supplementary Fig. S4). The MYB proto-oncogene, originally found as the retroviral oncogene myeloblastosis B, is a driver not previously identified, with predominant focal deletions affecting CDKN2A and duplications affecting BCL3 and MYB (Supplementary Fig. S4). The MYB proto-oncogene, originally found as the retroviral oncogene myeloblastosis B, is a driver not previously identified in OACs through SNV and CN analyses. Duplications overlapping MYB span the gene and the evidence of ecDNA events in four patients (CN = 5–45) support its role as an oncogene as identified in other cancer types.

Of the candidate genes identified, 10 were already known as OAC drivers, leaving 51 candidate SV OAC drivers (Fig. 4c, Supplementary Data 11). We classified each rearrangement using ClusterSV as simple (a single rearrangement not belonging to a cluster) or complex (multiple rearrangements forming a cluster) and the type of alteration. To accurately estimate the prevalence of SVs in individual drivers, we used a two-proportions-z-test.
of rearrangement overlapping with each gene, we identified intra-chromosomal regions spanning each pair of breakpoints and the genes lying within each region. This was done to capture oncogenes which are generally comprised within breakpoints in SVs that lead to amplification (i.e., DUPs, INVs or BNDs) or deletion (mainly DELs). A clear pattern emerged where simple alterations affected tumour suppressors genes including CDKN2A, ARID1A, SMAD4 and RUNX1, while complex clusters tended to affect oncogenes (ERBB2, CDK6, GATA4, GATA6) often involving amplifications (Fig. 4c). In addition, breaks within known tumour suppressor genes CDK12, ZNF21 and RNF43 were observed (Figs. 2c, 4c) and have been shown to result in loss in function (Supplementary Fig S4).

We curated genomic regions identified in our SV driver analysis without COSMIC genes and identified an additional 15 genomic bins containing 31 putative driver genes. These genes overlap with several OAC specific driver genes (GATA6, MUC6) previously identified14. In addition, drivers reported in other cancer types (PVT1, THADA and YES1) and ion channel genes (CACNG1, CACNG4, CACNG5, KCNB1, KCNS2, KCNK6) were identified to be preferentially affected by SVs (Supplementary Data 12).

RUNX1 is frequently disrupted by internal deletion of exons. RUNX1 was a candidate for a recurrent OAC driver (24% of patient samples, 92/383), uniquely affected by SVs, a known target of inter-chromosomal translocations in leukaemias, that has been shown to play a role either as an oncogene or TSG in a variety of cancer types35–37. It was previously reported as commonly deleted in OAC10,38, with a likely role as a tumour suppressor39,40.

RUNX1 was most commonly affected by simple SVs (60 patients) while 32 patients had complex SVs. The simple SVs

Fig. 2 Complex SVs leading to amplification of oncogenes. a Recurrent amplicons detected by Amplicon Architect associated with known OAC oncogenes. The number of tumours with detected amplicon is shown above. Y-axis showing copy number of segments spanning each gene, averaged along the length of segment. b Correlation of gene expression (TPM) and copy number of amplicons. c Example of an amplified region spanning CDK12, ERBB2, STAT3 and STAT5B, resembling ecDNA and d Reconstructed amplicon as an extrachromosomal circle containing ERBB2 and a CDK12-STAT5B fusion. e An amplified region spanning EGFR and joining chromosomes 7 and 13, forming an ecDNA and reconstructed as a circle (f).
comprised deletions (n = 53 events), duplications (n = 14) and inversions (n = 1) (Fig. 5a, Supplementary Data 11). To understand the biological effects of the RUNX1 deletions, we used data obtained from GTEx and RUNX1 isoform expression in our cohort (Supplementary Fig. 5A) to identify the most expressed transcript (ENST00000344691) for the RUNX1 locus and showed that the most frequently deleted regions encompassed one or more of three features: an enhancer element (chr21:36250083-36262951, 65 patients), three exons (ENSE00002454902, ENSE00003519701 and ENSE00001380483, 61 patients) that code for the Runt DNA binding domain, and the promoter 2 sequence (58 patients) (Fig. 5a, Supplementary Data 13). The loss of expression of the deleted exons 1–4 were observed significantly in transcriptomic sequencing compared to unmutilated tumours (Fig. 5b, Supplementary Fig. S5B). In addition, we observed that patients with promoter 2 loss have RUNX1 expression abolished while patients with exon deletions do not show significant difference in expression compared to unmutilated tumours (Supplementary Fig S5B, C).

We investigated the consequences of SVs for RUNX1, using PCR to confirm the genomic junctions, in 69 sequenced tumours as well as in two OAC cell lines, FLO-1 and OE3341. DNA was available for 17 tumours with a total of 22 RUNX1 SVs, and 20/22 (91%) were verified by PCR and Sanger sequencing, as were 3 SVs in the two cell lines (Supplementary Fig S5D, Supplementary Data 14).

Strikingly, many of the verified SVs were predicted to preserve the reading frame of RUNX1, and encode a protein with absent or modified Runt domain. Most of the individual verified SV calls, 18 of 23 (including 2 of 3 SVs in cell-lines), were internal deletions or duplications that removed or duplicated exons; at least 17 of these 18 were predicted to preserve reading frame; and 15 would encode a protein with absent or modified Runt domain.

**Discussion**

In this study, we identified rearrangement signatures and processes that shape the mutational and structural landscape of OAC. These encompass known DNA damage related processes including replication stress, complex rearrangements and a signature of unknown aetiology, associated with ME insertions. We estimated the contributions of ME insertions to the signature as multiple processes can result in clustered inter-chromosomal junctions. By assigning the clusters of inter-chromosomal junctions back to RS4, we found that 59% of clusters had evidence of ME insertions within the cluster. The reactivation of ME has been observed in multiple cancer types and previously been shown to
associated with amplifications and deletions, most notably in CDKN2A and BFB events. We found that ME activity in our cohort was mainly of the Plinian type leading to a large number of retrotranspositions. Recently, expression of transposable elements has been associated with DNA damage and immune response in cancer with possible implications for targeted therapies in OAC.

Complex rearrangements were shown to be prominent in OAC in previous studies and we estimated the contributions of ME insertions and ecDNA amplicons in generating complex rearrangement clusters. The evidence of BFB cycles and ecDNA accounting for 22% of complex clusters overlapping oncogenes suggest that it is a frequent process resulting in amplifications in OACs that can undergo selection. The high copy number and expression of these amplicons, observation of enhancer hijacking and the co-amplification of multiple cancer associated genes point to a potent mechanism of tumorigenesis, often with well-known oncogenes affected. Recently, mechanistic studies have shown that of telomere loss and chromosome bridge formation, generates BFB and micronuclei in in vitro systems. We speculate that ecDNA can arise from multiple mechanisms in OAC including chromosome bridge formation and via the episomal model that explains the wide variety of BFB-linked and non-BFB linked ecDNA we observed in this study.

In addition to SV-driven CN gains or losses, we identified the contribution of SVs to the mutational burden of known OACs.

Fig. 4 Recurrence and density of SVs in 1 Mb genomic bins. Scatter plot showing recurrence, the number of patients with an SV break in each 1 Mb bin (y-axis) and density, the average number of SV breaks in the bin over all tumours (x-axis). Bins are labelled with genes or fragile sites that they overlap: black, fragile sites; purple, intervals of amplification and deletion; red, putative genes under selection. Manhattan plot showing 1 MB bins containing putative drivers (red) and fragile sites (black) and genes coloured by methods discovered: Glodzik model adjusting for genomic context (Black), Focal (F, blue) and both methods (brown: FG). Oncoplot showing candidate driver genes identified using focal and Glodzik methods and annotated if each gene was found in Frankell et al. (2019).
drivers that would be recurrently affected by rearrangements, compared to SNVs and INDELs. We adopted conventions from TCGA and ICGC to annotate SV-affecting exons in canonical transcripts of each driver gene for a conservative estimate. Notably, a substantial number of SVs encompass exons, however more work is needed to identify alterations that have strong functional effects such as a loss of protein function seen in RUNX1. The large overlap of candidate driver events with CN gains and losses provides a reliable way to identify patients with driver gene alterations, but poses challenges in the clinical interpretation of copy neutral variants due to inversions and translocations.

Our results suggest that, for heavily rearranged tumours, current approaches based on targeted gene panels may miss a substantial number of driver gene alterations despite inclusions of large deletions and amplifications and more work is required to identify events that are clinically relevant. For the driver genes affected by SVs, we observe that 37% are affected by gains, 33.3% by losses, and 4.9% by fusions involving a driver gene in either fusion partner. In addition, fusions are more likely to be associated with copy number gains (3.8%), compared to copy neutral fusions (0.4%) and losses (0.7%). The increased frequency of fusions associated with gains is likely influenced by the rearrangement process generating the SV, such as the formation of ecDNA. Overall, our findings are in keeping with the literature which suggest that fusions in OAC are rare events and few are targetable or clinically relevant.

It remains the case that there are substantial challenges for identifying and prioritizing driver genes within SVs including: (1) gene dosage effects are hard to estimate as complex CN changes such as whole genome doubling are present in the majority of tumours; (2) complex SVs affecting driver genes can encompass large regions with multiple passenger genes implicated; and (3) downstream effects of SV events are hard to determine and need
to be validated experimentally. In our analysis we focused on driver genes in OAC and other cancers, as these genes often coincide with the recurrence of SVs within a large genomic region. We used additional evidence such as the patterns of SVs, focal deletions and duplications spanning the gene to identify the driver gene affected by SVs.

We have further identified regions in the genome with a high recurrence or density of SVs that were likely to undergo selection. The analysis recapitulated driver genes identified previously in OAC and pan-cancer studies13,14. Several drivers including AKA9, CDK12, RARA, CCR7 were associated in co-amplification of regions that were part of BFF and ecDNA while MYB was identified in breast and OAC to be affected by amplification in coding regions. BCL3 has been previously identified as a transcriptional activator in leukaemias and has recently been shown to activate an array of pathways including WNT and NFκB17. RUNX1 was mainly affected by CN loss and rearrangements.

Our analysis of RUNX1 rearrangements suggests that the most frequent events in OAC either result in promoter loss or remove or duplicate internal exons, so that a RUNX1 protein would still be encoded but with the RUNT domain disabled—the domain that mediates DNA binding and heterodimerisation with other transcription factors35. Although RUNX1 mutations and rearrangements have been described in OAC39,48,49 and other cancers, as these genes often be encoded but with the RUNT domain disabled. We have further identified RUNX1 mutations and rearrangements have been described in OAC39,48,49 and other cancers, as these genes often

Classification of SV footprints and rearrangement signature analysis. We classified SVs into footprints by identifying clusters as described using the ClusterSV R package (https://github.com/cancerit/ClusterSV). In addition, clusters of ME translocations were defined as clusters containing BNDs with at least one breakpoint differing with ME insertions. We then set aside with ME footprints and classified the remaining SVs as simple or complex rearrangements after excluding centromere and telomere regions.

Rearrangement signatures (RS) were extracted using the Palmpeset 1.0 R package50,51. Palmpeset was run for 1000 iterations for from 2 to 10 signatures, and signatures were selected based on coverage and similarity to signatures from cancers. We matched the extracted signatures to reference rearrangement signatures24 from Signal (https://signal.mutationsignatures.com). We clustered patients based on the exposures of the extracted SV signatures using the ConsensusClusterPlus25 (v1.46.0) R package. The final number of clusters (K) was chosen using the calcICL function with the K = 6 selected, based on the highest mean consensus score.

Chromothripsis, extrachromosomal amplicons and break-fusion-bridge events. Chromothripsis was identified as complex SV events with oscillating copy number changes, using ShatterSeek v0.4.21, and classified as high confidence (≥7 segments with oscillating copy number) or low confidence (4–6 segments) as recommended. Tumors resembling extrachromosomal amplifications or bridge–fusion–bridge cycles were identified using AmpliConArchitect v1.215: amplification-synthetic and synthetic-gene fusion events. This script was run using the clusterSV model to identify extrachromosomal regions with driver gene amplifications and identified breakpoints.

Rearrangement signature features and regression. To identify features associated to each RS, we carried out logistic regression using the glm function in R (stats R package) based on the presence of each RS as response and predictors including: number of ME insertions, chromothripsis events, complex SV clusters, mutations attributed to SNV signatures extracted using SigProfilerExtractor v1.1.056 listed by the COSMIC database57, mutational signature subtypes58, total BFF or ecDNA events and gene expression of known driver genes (Supplementary Data 3).

We log transformed and scaled the counts from the predictors. For each RS, predictors from the univariate analysis with p < 0.05 were used to build a multivariate model, refined with stepAIC (MASS R package, version 7.3-51.1) and FDR correction was done on the final model. In addition, we carried out a hold-out validation of 10 replicates including each of the cases with each signature and observed that positive associations between RS4-ME events, RS9-DRR subtype, RS6 + 12-BBF, RS7–SBS17a and negative associations between RS1-ec_CDK6, RS7-ME events were robust throughout each hold-out validation (Supplementary Data 16). A separate logistic regression model was built using the RNA-Seq gene expression profiles in SV driver genes (Supplementary Data 4, 11).

Methods

Study design, cohort selection and sequencing. Endoscopic biopsies and resection specimens were collected prospectively from 383 oesophageal adenocarcinoma patients, including 83 cases previously included in the ICGC pan-cancer (PCAWG) studies3. Patients were predominantly male (n = 329, 86%), with a median age at diagnosis of 66.8 years (IQR: 59–73.6), and presented at an advanced stage (T3N2 = 56.15%, T3N1 = 47.12%). All cases had an estimated tumour purity of >70%, following expert pathological review and underwent whole genome sequencing by Illumina using 100-150 bp paired end reads with 50-fold coverage. Regions resembling extrachromosomal amplifications or bridge–fusion–bridge cycles were identified using AmpliConArchitect v1.215: amplification-synthetic and synthetic-gene fusion events. This script was run using the clusterSV model to identify extrachromosomal regions with driver gene amplifications and identified breakpoints.

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Structural variation calling and validation. SVs were called, after alignment with bwa-mem to GRCh37hg19 (1000 Genomes Project Human, glk_v37 with decoy sequences hs37d5), using MANTA v0.277, as junctions that resembled deletions (DEL), inter-chromosomal junctions (BND), duplications (DUP), or inversions (INV). We discarded SVs that had any supporting reads in the matched normal;
In addition, we carried out a correlation matrix analysis on the response and predictors using the ccors function (Hmisc R package, version 4.2-0) and carried out FDR corrections for the final realizations. All associations except for R9-DDR, R9-Mutagenic, R9-CCNE1 and R2-KEF5 were validated using the correlation matrix analysis (Supplementary Data 4).

**Estimating the contributions of SV in known drivers.** To estimate the contributions of SV in recurrent drivers, we defined regions between two SV breakpoint called by MANTA and identified SVs with regions that overlapped exons in known driver genes from Frankell 2019 and Campbell 2020. To identify gene isoforms that are likely affected, we used annotations (vcf2maf tool, isoformOverrides_uniprot) from TCGA to select for overlaps in exons present in canonical transcripts of each gene. The predominant isoforms for RUNX1 were obtained from the GTEX database using the oesophagus mucosa and stomach tissue types.

In addition, GISTIC 2.033 was used to identify gains, amplifications, loss or deletions in genes in addition to SNVs, INDELS and SV.

**Identifying regions of frequent SVs.** We divided the genome into 1 Mb bins with 500 kb overlap and calculated breakpoint recurrence, i.e. the number of patients with at least one breakpoint in the bin, and breakpoint density, the average number of breakpoints in each bin across all samples (Fig. 3a).

To estimate the background SV rate19,32, we modelled breakpoint recurrence in each bin as a negative binomial linear regression, adjusted for the genomic context of each bin: fragile sites, copy number aberrations, GC content, replication timing49, histone methylation marks (H3K36me3 and H3K27ac), DNaseI hypersensitivity, and ALU sequences45. Bins were identified as being significantly recurrently altered if the residuals were ±2 standard deviations from the mean (Supplementary Data 10).

In order to further characterize bins that may reflect hotspots for SV activity we filtered bins that reflect known fragile sites and high-density regions (434/5597 bins). We then apply three methods to identify focal hotspots and select bins found by at least two methods: (1) model the per-bin SV counts genome-wide under a negative binomial distribution identifying the residual outliers as significant bins, (2) model the per-bin SV counts per-chromosome to account for chromosomal context, and (3) a rank-sum approach where counts are ranked per-patient and summed across each bin and significant bins identified via k-mers clustering.

To identify driver genes enriched in tumours with RS4 compared to tumours devoid of MEs (RS7 enriched tumours), we calculated the frequency of tumours with SVs in 1 Mb bins in RS4 and RS7 tumours (93 and 76, respectively) and identified those with a frequency difference of ≥15% between groups. We excluded fragile sites for this analysis.

**Statistics and reproducibility.** Statistical tests were carried out using R 3.5.3, with the wilcox.test function for the Wilcoxon rank sum test to associate biological features and each RS (n = RS9:261, RS1:180, RS7:266, RS4:283, RS2:335, RS6a:12264) and between RS patient groups (n = RS2:3129, RS7: 9106, RS6a:1274, RS4:232, RS1:42). All reported Wilcoxon rank sum tests p-values are two tailed. Permutation tests for the enrichment of the H3K27AC enhancer elements in ecDNA regions were carried out using the regionR30 package and overlapPermTest function with 5000 permutations. Two proportions z-test to compare recurrence of driver genes (n = 383) was carried out using the prop.test function with the alternative = ‘greater’ parameter, followed by multiple testing correction using p.adjust (method = ‘fdr’).

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

The sequencing data included in this study have been submitted to European Genome-phenome Archive (EGA; https://ega-archive.org/) under the accession numbers EGAD00001007808 (WGS) and EGAD00001007809 (RNAseq), respectively.

**Code availability**

R scripts used in the analyses are available on GitHub (https://github.com/fitzgerald-lab/Rearrangements-in-OAC).

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Author contributions
R.C.F., P.A.W.E. and G.C. conceived of the study. A.W.T.N., G.C., S.K., G.D. and J.S. carried out the analyses of the genomic data and statistical analyses. G.C., P.A.W.E., R.H. and S.A. designed and carried out the validation experiments. R.C.F., P.A.W.E. and S.T. supervised the research. G.D., J.M.W. and M.D.E. developed and maintained the genomics pipelines processing the WGS and RNA-seq data. A.M.R. coordinated the data collection. R.C.F. and S.T. obtained funding. R.C.F., A.W.T.N., P.A.W.E. and G.C. wrote the manuscript. All authors approved the manuscript.

Competing interests
R.C.F. has devised an early detection technology called Cytosponge, this device technology and the associated TFF3 biomarker is licensed to Covidien GI solutions (now owned by Medtronic) by the Medical Research Council. R.C.F. and M.O. are named inventors on patents pertaining to the Cytosponge and associated technology. R.C.F. is a shareholder of Cyted Ltd., a company working on early detection technology. R.C.F. has received consulting and/or speaker fees from Medtronic, Roche and Bristol Myers Squibb. The other authors declare no competing interests.

Additional information
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