**Supplementary data**

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| **Supplementary Table S1. The primers of candidate eccDNAs** | | | |
| Candidate eccDNAs | Forward primer | Reverse primer | Product size |
| O1 (chr3:153320590-153321556) | 5’-ACTCTGGCTGGTAGGACCAA-3’ | 5’-GATTGGGCAAAGAGGTGGGA-3’ | 256bp |
| O2 (chr14:65484268-65484970) | 5’-GGGCCTTTCATTGGAAGCAATC-3’ | 5’-TGACATTGCCCCAGAAGCG-3’ | 679bp |
| O3 (chr1:201630725-201631049) | 5'-GAGTGCTGCATCTAGCGGAG-3' | 5'-TGGGAGCTGGGGAGTCTAAT-3' | 153bp |
| O4 (chr5:167233287-167234692) | 5’-AAAAGCCTGGCCTCCATTGT-3’ | 5’-GCTGGGGGCCATCTTCATTA-3’ | 677bp |
| O5 (chr3:137490587-137491130) | 5’-GTAAGCAGGATCCACCCGAG-3’ | 5’-CTCGCGTCTATGCGGGTATT-3' | 278bp |
| S1 (chr8:38223231-38223598) | 5'-CGTTCACTCTGTTTCAGAGGCTT-3' | 5'-CCAGCCTCTCTATATGACTGATTCC-3' | 320bp |
| S2 (chr1:68141078-68144108) | 5'-TGGCCCACATCTAAGACCAG-3' | 5'-GGTCCGTCCTTACTGCTGTG-3' | 300bp |
| S3 (chr10:70277601-70277938) | 5'-ACGGAGAGCTATAATTGTGCGG-3' | 5'-CCTCCAGCCTTAGTCTCTGAGT-3' | 312bp |
| S4 (chr14:31640553-31641482) | 5'-AGTTCCAAAAGCAGAGGCCC-3' | 5'-CCATGCCATCTCCATCCACT-3' | 657bp |
| S5 (chr13:41890332-41891079) | 5'-GCAAGATGATTCTGTCGAACCCA-3' | 5'-ATGGAGCAACTTCTCACAGCATC-3' | 659bp |
| ***Notes: Ox or Sx indicates the up-regulated or down-regulated candidate eccDNAs in ESCC relative to matched normal epithelium. In the form of ChrX: Y-Z, X indicates chromosome id, Y indicates start site and Z indicates end site.*** | | | |

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| **Supplementary Table S2. The summary of reads and eccDNA count in each sample** | | | | | | |
|  | C1 | C2 | C3 | N1 | N2 | N3 |
| Raw reads | 141,510,152 | 138,644,094 | 148,054,446 | 136,927,808 | 137,162,048 | 142,572,110 |
| Clean reads | 141,495,966 | 138,630,264 | 148,034,092 | 136,914,164 | 137,144,340 | 142,555,544 |
| EccDNA count | 91,030 | 103,663 | 40,755 | 52,896 | 71,261 | 60,837 |
| ***Note: Cx indicated ESCC, while Nx indicated matched normal epithelium*** | | | | | | |

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| **Supplementary Table S3. The genes giving rise to more than 100 eccDNAs** | |
| The gene | The number of eccDNAs generated by the gene |
| LSAMP | 192 |
| CSMD1 | 159 |
| MACROD2 | 149 |
| MAGI2 | 142 |
| CNTNAP2 | 138 |
| RBFOX1 | 129 |
| WWOX | 125 |
| NRXN3 | 121 |
| LPP | 116 |
| NAALADL2 | 114 |
| DAB1 | 108 |
| AUTS2 | 106 |
| LRP1B | 106 |
| SHANK2 | 104 |
| PTPRD | 103 |
| IMMP2L | 101 |

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| **Supplementary Table S4. The genes generating the most candidate eccDNAs at differential level (Top 10)** | | | |
| The up-regulated eccDNAs in ESCC | | The down-regulated eccDNAs in ESCC | |
| The gene | The count of eccDNAs generated by the gene | The gene | The count of eccDNAs generated by the gene |
| AUTS2 | 13 | LSAMP | 7 |
| SHANK2 | 11 | CSMD1 | 7 |
| MACROD2 | 11 | BICD1 | 7 |
| ASIC2 | 10 | KALRN | 6 |
| LPP | 10 | ROBO2 | 6 |
| EYS | 10 | FHOD3 | 5 |
| MAGI2 | 9 | GRID2 | 5 |
| MYO3B | 9 | DOCK3 | 5 |
| TENM4 | 9 | MACROD2 | 5 |
| DMD | 9 | RUNX1 | 5 |

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| **Supplementary Table S5. The nucleotide pattern flanking the eccDNAs junctional sites** | | | | | | | | |
|  | Start position | | | | End position | | | |
| A | C | G | T | A | C | G | T |
| -10 | 0.297100624739 | 0.195207984525 | 0.215521492005 | 0.29215906197 | 0.293638279773 | 0.201812990025 | 0.214827939336 | 0.289720790867 |
| -9 | 0.292950145484 | 0.193176091939 | 0.2083150463 | 0.305547879517 | 0.293389034282 | 0.197109835986 | 0.217959763108 | 0.291541366624 |
| -8 | 0.287894796729 | 0.18256148507 | 0.225063259589 | 0.304469621851 | 0.289585331361 | 0.191631853574 | 0.210655786559 | 0.308127028506 |
| -7 | 0.265549396663 | 0.188521703322 | 0.210287336704 | 0.335630726551 | 0.285375249923 | 0.182160524933 | 0.223860379178 | 0.308603845966 |
| -6 | 0.317939715102 | 0.16721121388 | 0.227078897035 | 0.287759337224 | 0.276971342187 | 0.177397768711 | 0.220717718645 | 0.324913170457 |
| -5 | 0.376680375169 | 0.157230557497 | 0.216854413542 | 0.249223817032 | 0.332997393759 | 0.15799996749 | 0.242380402802 | 0.266622235949 |
| -4 | 0.390210070601 | 0.140444415546 | 0.230172792146 | 0.239161884946 | 0.378062062127 | 0.148387760963 | 0.224304686357 | 0.249245490553 |
| -3 | 0.301646645752 | 0.160237758524 | 0.271016542315 | 0.267088216649 | 0.358810557172 | 0.157219720737 | 0.237866892071 | 0.24610283002 |
| -2 | 0.240586918946 | 0.217271628819 | 0.239313599593 | 0.302817015881 | 0.267207421014 | 0.203926158314 | 0.257047958083 | 0.271818462589 |
| -1 | 0.274988215023 | 0.217271628819 | 0.246140758682 | 0.25012868653 | 0.235764560542 | 0.240966205563 | 0.233689320914 | 0.289579912981 |
| 0 | 0.282156732067 | 0.264162291325 | 0.226845906685 | 0.226835069924 | 0.287981490813 | 0.234567098512 | 0.226119843734 | 0.251331566941 |
| 1 | 0.247706670568 | 0.250513391527 | 0.161890364494 | 0.339889573411 | 0.279913522652 | 0.262574705917 | 0.17736525843 | 0.280141094621 |
| 2 | 0.239443640718 | 0.219401052249 | 0.146632205768 | 0.394523101264 | 0.248281018872 | 0.241096246688 | 0.145597295145 | 0.365020020915 |
| 3 | 0.24278678132 | 0.24751160888 | 0.151698391283 | 0.358003218518 | 0.250665106173 | 0.214844194477 | 0.152354015291 | 0.382131265679 |
| 4 | 0.320204598037 | 0.226146935635 | 0.169188922663 | 0.284459543664 | 0.273064690042 | 0.225047004448 | 0.161034260418 | 0.340848626712 |
| 5 | 0.316303364272 | 0.226699610418 | 0.181342349518 | 0.275654675791 | 0.325314130594 | 0.210991726133 | 0.183628905975 | 0.280059818918 |
| 6 | 0.30845213132 | 0.212774373229 | 0.191252566958 | 0.287520928494 | 0.313252816203 | 0.218274029162 | 0.183141251754 | 0.285326484501 |
| 7 | 0.293638279773 | 0.219265592744 | 0.197380754997 | 0.289715372487 | 0.306658647464 | 0.208510107988 | 0.19013096225 | 0.294694863917 |
| 8 | 0.287856868068 | 0.217683425717 | 0.202262715584 | 0.292196990632 | 0.294120515613 | 0.211750299366 | 0.196806406693 | 0.297317359948 |
| 9 | 0.284107348949 | 0.214383632157 | 0.206163949349 | 0.295345069545 | 0.292982655765 | 0.212481780696 | 0.19673596775 | 0.297794177409 |
| 10 | 0.282693151709 | 0.213148241465 | 0.212714771046 | 0.29144383578 | 0.294911599127 | 0.209929723608 | 0.199217585895 | 0.29593567299 |
| ***Note: The left column indicate the position of upstream (negative number) or downstream (positive number) of start/end site. The number in the main text indicates the percentage of one nucleotide at certain position.*** | | | | | | | | |