Extrachromosomal DNA is associated with decreased immune cell infiltration and antigen presentation, represents a potential cancer immune evasion mechanism

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Abstract

Extrachromosomal DNA (ecDNA) is a type of circular and tumor specific genetic element. EcDNA has been reported to display open chromatin structure, facilitate oncogene amplification and genetic material unequal segregation, and is associated with poor cancer patients' prognosis. The ability of immune evasion is a typical feature for cancer progression, however the tumor intrinsic factors that determine immune evasion remain poorly understood. Here we show that the presence of ecDNA is associated with markers of tumor immune evasion, and obtaining ecDNA could be one of the mechanisms employed by tumor cells to escape immune surveillance. Tumors with ecDNA usually have comparable TMB and neoantigen load, however they have lower immune cell infiltration and lower cytotoxic T cell activity. The microenvironment of tumors with ecDNA shows increased immune desert, decreased immune enriched fibrotic types. Both MHC class I and class II antigen presentation genes' expression are decreased in tumors with ecDNA, and this could be the underlying mechanism for ecDNA associated immune evasion. This study provides evidence that the presence of ecDNA is an immune escape mechanism for cancer cells.

60 Introduction

61 The immune system plays a crucial role in the protection and fight against
 62 cancer cells^{1,2}. Immunoediting, which includes three temporally distinct stages,
 63 termed elimination, equilibrium, and escape, has been proposed to explain the
 64 interactions between cancer cells and the immune system during the evolution
 65 of cancer³⁻⁵. The mechanisms responsible for the escape of tumor cells from
 66 immunosurveillance are not fully understood. Potential tumor intrinsic immune
 67 evasion mechanisms include: impaired antigen presentation machinery (such
 68 as B2M mutation, decreased antigen presentation gene expression⁶⁻⁸),
 69 overexpressed immune checkpoints or their ligands such as programmed
 70 death-ligand 1 (PD-L1) on cancer cells⁹. In addition, secreting of immune
 71 inhibitory cytokines, such as TGF- β , remarkably reshape the tumor immune
 72 microenvironment^{10,11}.

73

74 Extrachromosomal DNA (ecDNA) is a type of tumor specific DNA element that
 75 is circular and about 1–3 Mb in size. Since the 1960s, double minute
 76 chromosomes have been observed in the metaphase spreads of human
 77 cancer cells¹². Later these DNA elements without centrioles and telomeres are
 78 found to be circular, a few Mb in size, and their size but not their number is
 79 stable during the proliferation of cancer cells¹³. With the recent advance of
 80 sequencing and bioinformatics techniques, ecDNA has been found to be
 81 prevalent in various types of cancers, however ecDNA is rarely detected in
 82 normal tissues, suggesting the presence of ecDNA is a specific feature for
 83 some cancer cells¹⁴. EcDNA promotes accessible chromatin (open chromatin)
 84 formation, facilitates oncogene amplification, drives genetic heterogeneity, and
 85 is associated with poor prognosis in multiple types of cancer¹⁵⁻¹⁷.

86

87 Somatic DNA alterations are major determinants of cancer phenotypes,
 88 including immune phenotypes. EcDNA formation is a type of somatic DNA
 89 alteration. We hypothesize that ecDNA formation could be one mechanism for

90 cancer cells to evade immune surveillance.

91

92

93 **Results**

94 **EcDNA and tumor immune cell infiltration status**

95 For this study, we select cancer patient samples with both WGS and gene
96 expression data for analysis. The status of ecDNA in specific samples was
97 determined based on WGS data as previously described¹⁷. In total, 1684
98 samples with ecDNA status and gene expression information are available for
99 analysis (Supplementary Fig. S1).

100

101 First we investigate the correlation between the presence of ecDNA and tumor
102 immune infiltration status. The immune infiltration status was determined using
103 gene mRNA expression data. Multiple methods have been applied in the
104 quantification of tumor immune status, including TIMER, CIBERSORT, Xcell,
105 MCPcounter, Quantiseq and Estimate¹⁸⁻²³. With different methods, tumors with
106 ecDNA consistently show significantly decreased immune scores (Fig. 1a-c
107 and Supplementary Fig. S2). Importantly, the cytotoxic T cell (CD8⁺) levels and
108 cytotoxic scores are significantly decreased in tumors with ecDNA (Fig. 1d and
109 Supplementary Fig. S3). The composition of different immune cells was
110 calculated using gene expression data with multiple different methods,
111 including marker gene-based methods (Xcell and MCPcounter) or
112 deconvolution-based methods (Cibersort, Timer, and Quantiseq). Multiple
113 types of immune cells including B cell, NK cell and T cell show significantly
114 decreased composition in tumors with ecDNA in TCGA pan-cancer dataset as
115 a whole (Fig. 2a), or in separate cancer types, such as STAD (Stomach
116 adenocarcinoma), SKCM (Skin cutaneous melanoma), HNSC (Head and neck
117 squamous cell carcinoma) (Fig. 2b and Supplementary Fig. S3).

118

119 **EcDNA and tumor immune typing**

120 Tumor immune typing was performed according to two known studies^{24,25}.
 121 Thorsson et al used consensus clustering based on scored immune
 122 expression signatures to cluster cancer samples into six immune
 123 subtypes—wound healing, IFN- γ dominant, inflammatory, lymphocyte
 124 depleted, immunologically quiet, and TGF- β dominant²⁵. In tumors with ecDNA,
 125 lymphocyte depleted type is up-regulated, while inflammatory and TGF- β
 126 dominant types are down-regulated (Fig. 3a). Bagaev et al used unsupervised
 127 dense Louvain clustering based on ssGSEA scores of 29 Fges (functional
 128 gene expression signatures) of immune and stromal related genes to cluster
 129 cancer samples into four distinct microenvironments: (1) immune-enriched,
 130 fibrotic (IE/F); (2) immune-enriched, non-fibrotic (IE); (3) fibrotic (F); and (4)
 131 immune-depleted (D)²⁴. In tumors with ecDNA, fibrotic immune-enriched type
 132 of TME (IE/F) is dramatically decreased, while immune desert type TME (D) is
 133 significantly up-regulated (Fig. 3b).

134

135 **EcDNA and tumor immune escape**

136 Expression of immune inhibitory immune checkpoint genes, such as PD-L1,
 137 CTLA4 is significantly down-regulated in tumors with ecDNA (Fig. 4a and
 138 Supplementary Fig. S4), suggesting the immune evasion of tumors with
 139 ecDNA is not through stimulating immune checkpoint signaling. This also
 140 implicates that immune checkpoint inhibitor therapy alone may not work in
 141 tumors with ecDNA.

142

143 **Antigen presentation and ecDNA mediated immune escape**

144 Tumors with ecDNA show decreased immune cell infiltration, suggesting a
 145 decrease of immunogenicity in ecDNA-containing tumor cells. The
 146 immunogenicity of tumor cells determines the tumor associated immune
 147 response, and the antigenicity encoded by neoantigenic mutations is an
 148 important determinant of tumor immunogenicity²⁶. Tumors with ecDNA show
 149 comparable TMB and neoantigen counts, suggesting a comparable

150 antigenicity (Fig. 4b and Supplementary Fig. S5). This implies that the
151 decreased immunogenicity of ecDNA-containing tumors was not caused by
152 impaired antigenicity.

153

154 Antigen presentation efficiency is another important determinant of tumor
155 immunogenicity²⁶. The function of MHC class I antigen presentation pathway is
156 to display peptide fragments of proteins from within the cell to cytotoxic T cells;
157 MHC Class II molecules are normally found only on professional
158 antigen-presenting cells such as dendritic cells, mononuclear phagocytes,
159 some endothelial cells, thymic epithelial cells, and B cells. The antigens
160 presented by class II peptides are derived from extracellular proteins.
161 Expression of antigen presentation related genes, including MHC I, MHC II
162 related genes, are compared between tumors with and without ecDNA (Fig. 5a
163 and Supplementary Fig. S6). In tumors with ecDNA significantly decreased
164 expression of MHC class I and class II genes are observed (Fig. 5a). Gene set
165 enrichment analysis indicates MHC class I and class II related genes are
166 significantly down-regulated in several cancer types (Fig. 5b). The impaired
167 expression of MHC I and II related antigen presentation genes could be the
168 mechanism underlying decreased immune infiltration in tumors with ecDNA.

169

170

171 Discussion

172 Here we provide evidence to show that the presence of ecDNA is associated
173 with decreased immune cell infiltration, decreased cytotoxic T cell
174 percentage/composition, decreased expression of both class I and class II
175 antigen presentation machinery genes. This analysis indicates that the
176 presence of ecDNA could be one of the mechanisms employed by tumor cells
177 to evade immune surveillance. EcDNA is preferentially detected in tumors, and
178 less frequently in cultured tumor cells²⁷. The immune selection pressure in
179 tumors could be the underlying mechanism for this observation.

180

181 This study is based on gene expression data derived from bulk tumor samples,
182 currently it is unclear if the gene expression differences happens in tumor cells
183 or in the microenvironment immune cells or stromal cells. Consistently
184 down-regulated antigen presentation related genes are observed in various
185 types of tumors with ecDNA, and the functional consequence of these gene
186 expression down-regulation in antigen presentation process need to be
187 examined using experimental assays.

188

189 Based on this study ecDNA could directly induce tumor immune escape
190 through down-regulating the expression of antigen presentation genes.
191 Currently there are no experimental evidences supporting the alternative
192 possibility that immunosuppressive microenvironment directly induces ecDNA
193 formation. Potential inducers for ecDNA formation include DNA repair defect
194 (like HRD), telomere shortening, cell cycle defects, and most of these ecDNA
195 inducers are cell-intrinsic defects.

196

197 The detailed molecular mechanism responsible for the decreased MHC class I
198 and II antigen presentation genes' expression, and immune evasion in
199 ecDNA-containing tumors is not clear. The ecDNA associated oncogene could
200 be a potential mechanism. The function of nuclear circular DNA on immune
201 response is unknown. Cytoplasmic DNA is known to stimulate immune
202 response through cGAS-STING pathway²⁸, and in tumors with ecDNA, this
203 pathway is not over-activated (Supplementary Fig. S7). EcDNA formation is a
204 type of genomic DNA copy number alteration, its detections with copy number
205 signature analysis could reveal potentially actionable biomarkers for cancer
206 precision therapy²⁹⁻³¹. Tumors with ecDNA are known to have poorer
207 prognosis compared with tumors without ecDNA¹⁷. Stimulating the antigen
208 presentation pathway could potentially revert the ecDNA-mediated immune
209 escape.

210

211

212 **Materials and Methods**

213 **Data Source**

214 EcDNA status information was determined using AmpliconArchitect from whole
215 genome sequencing (WGS) data as described previously¹⁷. Gene expression
216 data are available for the majority of the cancer genome atlas (TCGA) but not
217 pan-cancer analysis of whole genomes (PCAWG) datasets. For downstream
218 immune infiltration and gene expression analysis, we only keep TCGA
219 samples. Tumor immune cell infiltration information for TCGA samples was
220 downloaded from the TIMER webserver (<http://timer.comp-genomics.org/>),
221 including the results calculated by TIMER, CIBERSORT, quanTIseq, xCell,
222 and MCP-counter algorithms. Somatic mutation data detected by Mutect2 was
223 download from UCSC xena (GDC-PANCAN.mutect2_snv.tsv). The pan-cancer
224 gene-level RNA-Seq data of TCGA samples was downloaded from UCSC
225 xena, including counts and normalized transcripts per million (TPM) data.
226 Immune subtyping and tumor microenvironment (TME) information of TCGA
227 samples are based on reports of Thorsson et al and Bagaev et al study
228 respectively^{24,25}. The leukocyte fraction data of TCGA samples are based on
229 the results of Thorsson et al study²⁵. In the downstream analysis, we only
230 keep cancer types where the count of ecDNA samples was more than 20. All
231 methods were performed in accordance with the relevant guidelines and
232 regulations.

233

234 **Calculation of cancer immune scores**

235 In addition to immune cell infiltration quantification, we calculated a variety of
236 additional immune microenvironment quantitative scores. The
237 immunophenoscore (IPS) was used to measure the immune state of the
238 samples. IPS was based on the expression of major determinants, identified
239 by a random forest approach, and these factors were classified into four
240 categories: major histocompatibility complex (MHC) molecules, effector cells,
241 suppressor cells and checkpoint markers. We used R scripts and IPS genes
242 provided by the origin paper to calculate IPS scores³². ESTIMATE (Estimation
243 of STromal and Immune cells in MAlignant Tumor tissues using Expression
244 data) is a tool using gene signatures to generate three scores: stromal score,
245 immune score and estimate score, we used R package Estimate to calculate
246 the immune score²³. The cytolytic activity (CYT) score was a quantitative
247 means of assessing cytotoxic T cell infiltration and activity and was calculated

as the geometric mean of expression of *GZMA* and *PRF1* genes³³. The tumor inflammation signature (TIS) uses 18-gene signature to measure a pre-existing but suppressed adaptive immune response within tumors. The TIS has been shown to enrich for patients who respond to the anti-PD1 agent pembrolizumab. TIS was calculated by gene set variation analysis (GSVA) using the 18-gene signature mentioned by Danaher et al³⁴.

Tumor mutational burden (TMB) and neoantigen burden

TMB was defined as the number of non-synonymous alterations per megabase (Mb) of genome examined. We used 38 Mb as the estimate of the exome size: TMB = (whole exome missense mutations) / 38. Tumor neoantigen are generated by somatic mutations, and can be recognized as foreign by immune cells, conferring immunogenicity to cancer cells. Neoantigen was predicted based on somatic mutation and human leukocyte antigen (HLA) typing data. HLA typing data for TCGA cancer was obtained from Thorsson et al study²⁵. Mutect2 mutation files were first transformed into VCF format by maf2vcf tools, and we used NeoPredPipe to predict neoantigen³⁵. We only evaluated single-nucleotide variants leading to a single amino acid change, and novel peptides of nine amino acids were considered. From the output results, if the IC50 of a novel peptide is less than 50nM, and the TPM expression level is greater than 1, then this peptide is labeled as neoantigen. A mutation was considered neoantigenic if there was at least a single peptide produced from the mutated base that produce a neoantigen. Neoantigen burden was calculated similarly as TMB: (Total counts of neoantigens in the exome) / 38.

Gene set enrichment analysis (GSEA)

For each cancer type, we used Deseq2 to identify differentially expressed genes between ecDNA and non-ecDNA samples³⁶. Then gene set enrichment analysis was performed by using R package "fgsea". We downloaded gene list gmt file for the following pathways from MSigDB database, including "REACTOME_MHC_CLASS_II_ANTIGEN_PRESENTATION", "REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_PRESENTATION", "GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I", and "GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_OR_POLYSACCHARIDE_ANTIGEN_VIA_MHC_CLASS_II". The GSEA p values were corrected by FDR method, and was considered significant if less

287 than 0.05. For each cancer sample, we also calculated corresponding pathway
288 GSVA scores using R package “GSVA”³⁷.

289

290 **Statistical analysis**

291 All *P* values showed in boxplot were calculated by Wilcoxon tests using R. We
292 used the following convention for symbols indicating statistical significance: ns:

293 $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. Immune subtype

294 enrichment analysis was conducted by chi-squared test. All statistical tests and
295 visualization analyses were performed with R.

296

297

298 **Data Availability Statement**

299 Only publicly available data were used in this study, and data sources and
300 handling of these data are described in the Materials and Methods and in

301 Supplementary Table 1-3. All codes required to reproduce the results reported

302 in this manuscript are freely available at:

303 https://github.com/XSLiuLab/ecDNA_immune . Analyses can be read online at:

304 https://xsluolab.github.io/ecDNA_immune/ . Further information is available

305 from the corresponding author upon request.

306

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315

316 **Contributions**

317 TW, CW, collected the data and performed the computational analysis. XZ,

318 GW, WN, ZT, FC participated in critical project discussion. XSL designed,
319 supervised the study and wrote the manuscript.

320

321 **Conflict of interest**

322 The authors declare no competing interests.

323

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413 **Figure legends**

414 **Figure 1. ecDNA and tumor immune infiltration scores. a-d** Comparisons
415 of immune infiltration scores quantified by different methods between tumors
416 with and without ecDNA. **a** Estimate ImmuneScore; **b** XCELL Immune score; **c**
417 Leukocyte fraction, **d** MCPCounter cytotoxicity score. Wilcoxon test p values
418 are shown.

419

420 **Figure 2. ecDNA and the infiltration of different types of immune cells. a**
421 Comparisons of the compositions of different types of immune cells between
422 tumors with ecDNA and without ecDNA. The immune cell compositions have
423 been quantified by five different methods, including Cibersort, Xcell, Timer,
424 MCPcounter and Quantiseq. Wilcoxon test p values are shown. ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. **b** Comparison of immune cell
426 infiltration levels quantified by five different methods between ecDNA and
427 non-ecDNA samples in different cancer types. Heatmap color indicates ratio of
428 the median infiltration level for specific immune cell and specific cancer type
429 between ecDNA and non-ecDNA samples. TCGA cancer type acronyms:
430 STAD (stomach adenocarcinoma), SKCM (skin cutaneous melanoma), HNSC
431 (head and neck squamous cell carcinoma), LUAD (lung adenocarcinoma),
432 BLCA (bladder urothelial carcinoma), BRCA (breast invasive carcinoma),

433 ESCA (esophageal carcinoma).

434

435 **Figure 3. ecDNA and tumor immune typing.** **a** TME classification in tumors
436 with and without ecDNA according to Thorsson et al method. Chi-squared test
437 p value is shown. C1: wound healing; C2: IFN- γ dominant; C3: inflammatory;
438 C4: lymphocyte depleted; C5: immunologically quiet; C6: TGF- β dominant. **b**
439 Immune type classification in tumors with ecDNA and without ecDNA
440 according to Bagaev et al method. Chi-squared test p value is shown. D:
441 immune-depleted; F: fibrotic; IE: immune-enriched, non-fibrotic; IE/F:
442 immune-enriched, fibrotic.

443

444 **Figure 4. ecDNA and expression of inhibitory immune checkpoint genes**
445 **and TMB.** **a** Expression of inhibitory immune checkpoint genes in tumors with
446 ecDNA and without ecDNA. Wilcoxon test *P* values are shown. **b** Tumor
447 mutation burden (TMB) difference in different types of tumors with and without
448 ecDNA. Wilcoxon test p values are shown. ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$,
449 ***: $P \leq 0.001$, ****: $P \leq 0.0001$. TCGA cancer type acronyms: STAD (stomach
450 adenocarcinoma), SKCM (skin cutaneous melanoma), HNSC (head and neck
451 squamous cell carcinoma), LUAD (lung adenocarcinoma), BLCA (bladder
452 urothelial carcinoma), BRCA (breast invasive carcinoma), ESCA (esophageal
453 carcinoma), GBM (glioblastoma multiforme).

454

455 **Figure 5. ecDNA and antigen presentation genes' expression.** **a** mRNA
456 expression of MHC class I and class II antigen presentation related genes in
457 tumors with and without ecDNA. Wilcoxon test p values are shown. **b** GSVA
458 scores of MHC class I or class II antigen presentation genes in tumors with
459 and without ecDNA. Wilcoxon test *P* values are shown. ns: $P > 0.05$, *: $P \leq 0.05$,
460 **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$.

Figure1

Figure2

a

b

Figure3

a

b

Figure4

a

b