BBA - Reviews on Cancer 1874 (2020) 188392

Contents lists available at ScienceDirect

BBA - Reviews on Cancer

journal homepage: www.elsevier.com/locate/bbacan

Review

Classification of extrachromosomal circular DNA with a focus on the role of extrachromosomal DNA (ecDNA) in tumor heterogeneity and progression

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ARTICLE INFO

Keywords:
eccDNA
Extrachromosomal DNA
Biogenesis
Oncogene amplification
Micronuclei
Tumor heterogeneity

ABSTRACT

Although the eukaryotic genome is mainly comprised of linear chromosomal DNA, genes can also be found outside of chromosomes. The unconventional presence of extrachromosomal genes is usually found to be circular, and these structures are named extrachromosomal circular DNA (eccDNA), which are often observed in cancer cells. Various types of eccDNA including small polydispersed DNA (spcDNA), telomeric cirlces, microDNA, etc. have been discovered. Among these eccDNA, extrachromosomal DNA (ecDNA), which encompasses the full spectrum of large, gene-containing extrachromosomal particles, has regained great research interest due to recent technological advances such as next-generation sequencing and super-resolution microscopy. In this review, we summarize the different types of eccDNA and discuss the role of eccDNA, especially ecDNA in tumor heterogeneity and progression. Additionally, we discuss some possible future investigative directions related to ecDNA biogenesis and its clinical application.

1. Introduction

In 1869, Friedrich Miescher discovered DNA as the fundamental genetic element in most individuals and species [1]. In the eukaryotic nucleus, chromosomes consisting of linear double-stranded DNA and histone proteins are the major structures for storing genetic information [2]. However, genes can also be found on extrachromosomal elements. Similar to the structure of the bacterial genome, these DNA are also circular such as traditional mitochondrial DNA and chloroplast DNA [3,4]. Additionally, the presence of other types of circular DNA has been observed in nuclei and the cytoplasm [5]. Here, we have named these DNA extrachromosomal circular DNA (eccDNA), of which size ranges from tens to millions of bp (base pairs). Based on the different sizes and sequences, we further categorize eccDNA into small polydispersed DNA (spcDNA) (100 bp-10 kb), telomeric circles (t-circles) (multiples of 738 bp), microDNA (100–400 bp) and the largest one extrachromosomal DNA (ecDNA) (millions of bp) (Table 1). Under recent technological advances, the knowledge of the biological properties and functions of eccDNA has broadened, the interest in these extrachromosomal has been renewed and many previously undescribed extrachromosomal circular DNA have been observed [6]. In this review, we briefly summarize the different types of eccDNA and focus on ecDNA especially its crucial role in tumor heterogeneity and progression. We also review the possible formation and elimination mechanisms of ecDNA and discuss the clinical application and future investigation directions of ecDNA.

2. A brief history of the discovery of eccDNA

The discovery of eccDNA verified the idea that the DNA of higher

Abbreviations: eccDNA, extrachromosomal circular DNA; spcDNA, small polydispersed DNA; bp, base pairs; t-circles, telomeric circles; ecDNA, extrachromosomal DNA; DMs, double minutes; ALT, alternative lengthening of telomeres; ORC, origin recognition complex; WRN, Werner syndrome protein; HSRs, homogeneously staining regions; GBM, glioblastoma; FISH, fluorescent in situ hybridization; DHFR, dihydrofolate reductase; MTX, methotrexate; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; BFB, Breakage-fusion-bridge; HU, Hydroxyurea; HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; ctDNA, circulating tumor DNA

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https://doi.org/10.1016/j.bbcan.2020.188392
Received 19 May 2020; Received in revised form 26 June 2020; Accepted 10 July 2020
Available online 28 July 2020

Table 1
Classification of eccDNA.

Name of the eccDNA	Size	Frequency	Characteristic	Function	Main Refs
SpcDNA	100 bp- 10 kb	More frequent in unstable cells	Homologous to repetitive chromosomal sequences; byproducts or intermediates of gene rearrangement	Initiation or enhancement of genomic instability	[21,22]
Telomeric circles	Integral multiples of 738 bp	Especially in ALT cells	Duplex or single-stranded molecule composed of telomeric repeats	Involved in the ALT pathway and ALT+ tumors	[27]
MicroDNA	100–400 bp	More frequent in tumor cells	Origin from regions with high GC content and exon density	Circulating microDNA cancer liquid biopsy; biogenesis of microRNA	[39,41]
EcDNA	1–3 Mb	Tumor cells (especially in GBM)	Acentric, without telomere, containing full genes	Providing oncogene amplification and drug resistance	[51]

Abbreviations: eccDNA: extrachromosomal circular DNA; spcDNA: Small polydispersed DNA; ALT: alternative lengthening of telomeres; ecDNA: extrachromosomal DNA; GBM: glioblastoma.

organisms could also be organized in rings [7]. Yasuo Hotta and Alix Bassel found variable sizes of extrachromosomal circular DNA in boar sperm under an electron microscope [5]. These circular DNA were also seen in many other eukaryotic species, including yeast [8], *Drosophila melanogaster* [9], mice [10], hamsters [11] and human [12]. The majority of eccDNA found in normal cells is rather small and often less than 500 bp [10,12–15].

Contemporaneously, another type of eccDNA with a larger size was discovered in tumor cells by wild-field light microscopy. When investigating the mitotic abnormalities in surgically removed tumor samples stained by acetic orcein, Spriggs and Cox encountered a surprising new anomaly which was small chromatin bodies neighboring the ordinary intact chromosomes [16]. These bodies were named double minutes (DMs) because they are often observed in pairs. Spriggs's study further validated that these bodies were most frequently noticed in childhood malignant brain tumors [17]. By a buoyant density method, DMs were also found in HeLa cells [18].

With the help of Southern blots and sequencing, eccDNA was found to be homologous to genomic DNA [9,19]. Moreover, these elements were much more common in genetically unstable cells (e.g., tumor cells and cells with defective DNA repair system) than normal ones [20], suggesting that eccDNA could reflect the instability of the genome.

3. Classification of eccDNA

3.1. Small polydispersed DNA (spcDNA)

Before the 1980s, eccDNA was supposed to be rather small elements usually detected under an electron microscope [12]. At that time, spcDNA was thought to be the only component of eccDNA, thus the two terms were often used interchangeably. However, when many other larger extrachromosomal circular DNA types such as DMs were found by light microscopy, the term spcDNA was not appropriate to describe all of them. Therefore, spcDNA refers to those primarily found eccDNA that are between a few hundred bp to a few thousand bp and measured 0.1 μm to 2 μm [21]. SpcDNA is derived mostly from repetitive sequences organized in the genome [22].

Biogenesis: It is suggested that the chromosomal regions with a high level of direct tandem repeats facilitate spcDNA formation in the early embryo [23]. For their diversity in size, amount and sequence, chromosomal recombination and rearrangement, replicative mechanisms and transposition events were proposed for spcDNA formation (reviewed in [24]). Additionally, a DNA ligaseIV-dependent pathway was proposed [25].

Function: SpcDNA is a common element in a large variety of eukaryotic cells, but its quantity is limited [22]. It is much more abundant in genetically unstable cells and tissues, such as HeLa cells, colon carcinoma, fibroblasts of Fanconi Anemia and carcinogen-treated cells [20]. Therefore, the existence of spcDNA is a phenomenon associated with genomic instability. Autonomously replicating spcDNA may lead to alterations in chromosome structure and in gene expression patterns, which plays an active role in initiating or enhancing genomic instability [22].

3.2. Telomeric circles (t-circles)

Telomeres are the repetitive nucleotide sequences (like TTAGGG/CCCTAA in mammals) at the ends of linear DNA molecules that protect DNA from degradation and fusion [26]. A special group of eccDNA harboring only telomeric repeats is described as t-circles which are duplex or single-stranded and integral multiples of 738 bp in size [27].

Biogenesis: The formation of t-circles was proposed to either from intramolecular recombination between telomeric repeats or the excision from telomeric loops [27]. Telomeric loops are formed by the invasion of the single-stranded overhang of a telomere into the double-stranded region of telomeric repeats [28]. Once t-circles are formed, they can amplify autonomously via a rolling circle mechanism generating long extrachromosomal telomeric repeats which can be incised back to the chromosomal termini [29]. Several proteins have also been found related to the regulation of t-circles formation such as origin recognition complex (ORC) and Werner syndrome protein (WRN) [30,31].

Function: Telomerase can add de novo telomeric repeats onto chromosomal termini to compensate the telomere shortening caused by DNA replication [32,33]. Interestingly, t-circles were proved to play an important role in alternative lengthening of telomeres (ALT) [34]. The involvement of t-circles in ALT maintenance was first observed in the mitochondrial genomes of yeasts [35]. With its ability to serve as a template for rolling-circle telomeric DNA synthesis, a t-circle can provide efficient telomere lengthening. Additionally, ALT maintenance was estimated to account for 15% of the immortalization of human tumor cells, including osteosarcoma, renal cell carcinoma, breast carcinoma, etc. [36]. Among the different forms of t-circles, single-stranded $(CCCTAA)_n$ t-circles (named as C-circles) were found to be ALT+ tumors specific. The detection of C-circles (CC Assay) is proposed to be utilized in the diagnosis and management of ALT+ tumors [37].

3.3. MicroDNA

When investigating the genetic mosaicism during brain development, Shibata and his colleagues encountered a new group of eccDNA in mouse embryonic brains by electron microscopy [38], which they named “microDNA” on the basis of its tiny size. MicroDNA are short (100–400 bp) single and double-stranded circular DNA derived mostly from unique non-repetitive genomic sequences. Further sequencing analysis revealed that these elements exhibit short direct repeats of 2–15 bp at their 5′ and 3′ terminus, deriving from genomic regions with high GC content and exon density [39].

Biogenesis: Given its possible relationship with the homology-dependent repair pathway and mismatch repair pathway, multiple

mechanisms for the generation of microDNA are suggested. Single-stranded microDNA can be formed through the excision of a DNA loop caused by replication slippage, or the ligation of circularized nascent DNA after a DNA break or replication block. Double-stranded microDNA can be generated from microhomology-mediated circularization of the double-stranded DNA break fragments or converted from single-stranded microDNA (intensively reviewed in [39]).

Function: MicroDNA is too small in size to carry full protein coding genes, and the actual function of microDNA remains unclear. Paulsen used artificial molecules with known microDNA sequences and structure to mimic real microDNA, finding that it can express functional small regulatory RNA, including microRNA and novel small interfering like RNA [40], suggesting that microDNA could be transcribed in cells and regulate gene expression. In a recent study, cell-free microDNA from uniquely mapping regions had been detected in plasma and serum in ovarian cancer [41], further demonstrating that tumor cells could release specific microDNA into circulation. Of note these tumor-derived microDNA are significantly longer than normal. Thus, the detection of circulating microDNA might provide a new method for cancer liquid biopsy.

3.4. From double minutes to ecDNA

Back in 1965, DMs, which are absent of centromeres or telomeric ends, were first discovered in metaphase neuroblastoma cells [16] and subsequently found in various types of cancers [42–46]. Based on the Mitelman Database (the largest chromosome aberration database where all the patient cases were manually collected from the literature by Felix Mitelman and his colleagues), the overall frequency of DMs in primary cancers is 1.4% (787 DMs-positive cases relative to a total 54,398 cases) [47]. The database showed that DMs could be found in numerous types of cancers in which adrenal carcinoma (28.6%) and neuroblastoma (31.7%) rank the most. In addition, the frequency of DMs in malignant tumors is much higher than that in benign ones [48]. However, it is controversial that the total frequency of DMs in the database was much lower than that in previous reports [42–44], indicating the numbers might be underestimated.

Recently, with the combined applications of whole-genome sequencing, computational and cytogenetic analysis, Mischel and his colleagues suggested that the coverage of this group of eccDNA should be broadened [49], because they can either be detected in a double body form (like DMs) or a single body form, and DMs only make up 30% of these extrachromosomal elements [50]. The term ecDNA was used to describe the full spectrum of elements that are circular and 1–3 Mb in size. EcDNA contain more than one full gene and regulatory regions, lack a centromere and telomere and are visible under light microscope with DAPI staining [51]. EcDNA can be found in 46% of tumor cell lines (17 different tumor types, 2572 dividing cells) [49]. It is present in around half of glioblastoma (GBM), prostate, breast, lung, melanoma and renal tumor cells but is rare in colon and hematologic cancer. Additionally, patient-derived tumor cells exhibit higher ecDNA-positive rates than cell lines, suggesting that the in vivo tumor environment may somehow contribute to ecDNA maintenance [52].

Biogenesis: Several different models are proposed to illustrate the formation of ecDNA, including breakage-fusion-bridge (BFB) cycle [53], translocation-deletion-amplification model [54], “episome” model [55] and chromothripsis [56] (discussed in Section 5).

Function: As the residing genes are actively transcribed, ecDNA was proved to be euchromatin [57]. The intracellular location of ecDNA has also been discussed, and it localizes to the nuclear periphery during G1 then relocates to the nuclear interior and amplifies in early S phase [58,59]. Furthermore, oncogenes and drug-resistance gene amplification on ecDNA have been found, which emphasizes ecDNA's important role in tumor heterogeneity and progression [60–62].

4. EcDNA and gene amplification

4.1. The oncogene amplification in the form of ecDNA

Overexpression of oncogenes drives tumorigenesis [63]. As mentioned above, ecDNA is a common element in cancer cells. Its pivotal role in tumor progression might be closely related to gene amplification. Two abnormal chromosomal structures, ecDNA and homogeneously staining regions (HSRs), are thought to be the cytogenetic hallmarks of gene amplification in cancers [64]. HSRs are segments of chromosomes which exhibit intermediate intensity in trypsin-Giemsa preparation [65].

MYCN amplification was found both on ecDNA and HSRs in neuroblastoma cell lines [61]. Other genes have also been found amplified on ecDNA and HSRs in tumor cells, including c-myc, c-Ki-ras, mdm2, Rel, Abcg2, EGFR and Mll (reviewed in [24]). MYC amplification in seven tumor cell lines was precisely detected by an integrated approach including next-generation sequencing, single nucleotide polymorphism array, fluorescent in situ hybridization (FISH) and polymerase chain reaction-based techniques [66]. The proto-oncogene MET amplification was also seen by FISH on ecDNA in glioblastoma cells [67]. The software, AmpliconArchitect, was developed to reconstruct the fine structure of focally amplified regions, which provides clear insight into the oncogene amplification on ecDNA [68].

Comparing these two amplification sites, the amplified genes in cancer cells most frequently localize on ecDNA rather than HSRs [42,64]. Schimke hypothesized that the extrachromosomal ecDNA represented an unstable form of gene amplification while the chromosomal HSRs was a stable one [69]. Thus, ecDNA can be a major force driving tumor genomic instability.

4.2. EcDNA drives tumor drug resistance and heterogeneity

Another issue related to gene amplification on ecDNA is drug resistance. The dihydrofolate reductase (DHFR) gene copy number and the reductase synthesis in methotrexate(*MTX*)resistant tumor cells are many folds higher than in sensitive ones [60]. Interestingly, when the resistant cell lines are grown in the absence of MTX, the DHFR gene copies and dihydrofolate reductase are reduced. Other studies have reported that in both human and rat cell lines, the DHFR gene amplification occurred either on ecDNA mostly or HSRs [42,70]. Singer suggested that partial or complete loss of the parental chromosomes was able to initiate DHFR amplification on both ecDNA and HSRs in MTX-resistant human cell lines [71].

By investigating the drug resistance of targeted therapies, Nathanson and his colleagues moved a step further [72]. In glioblastoma, the epidermal growth factor receptor (EGFR) gene is frequently mutated as EGFRvIII, which can provide tumor cells with growth advantages [73]. They found that EGFRvIII is mostly amplified on ecDNA and makes the tumor cells more sensitive to tyrosine kinase inhibitor (TKI) treatment as well. Loss of ecDNA containing EGFRvIII results in the resistance of TKI at the sacrifice of a growth advantage. Under the treatment of TKI, a rapid decrease in EGFRvIII gene copies was noticed. More interestingly, the number of ecDNA containing EGFRvIII could increase again just after TKI withdrawal [72]. These examples indicate that ecDNA assists tumor cells in rapid environmental adaption and clonal evolution.

Tumor heterogeneity plays an important role in driving neoplastic progressions and therapeutic resistance [52]. The heterogeneity provides the tumor with a pool of genomic alterations that may help tumors to fit the environment under different pressures [74,75]. Genes amplified in ecDNA are considered to rapidly increase intratumoral heterogeneity possibly through its acentric structure and unequal segregation. During mitosis, in contrast to chromosomal DNA, ecDNA is segregated unevenly due to the absence of a centromere and thus the daughter cells contain different copy numbers of ecDNA. The cells

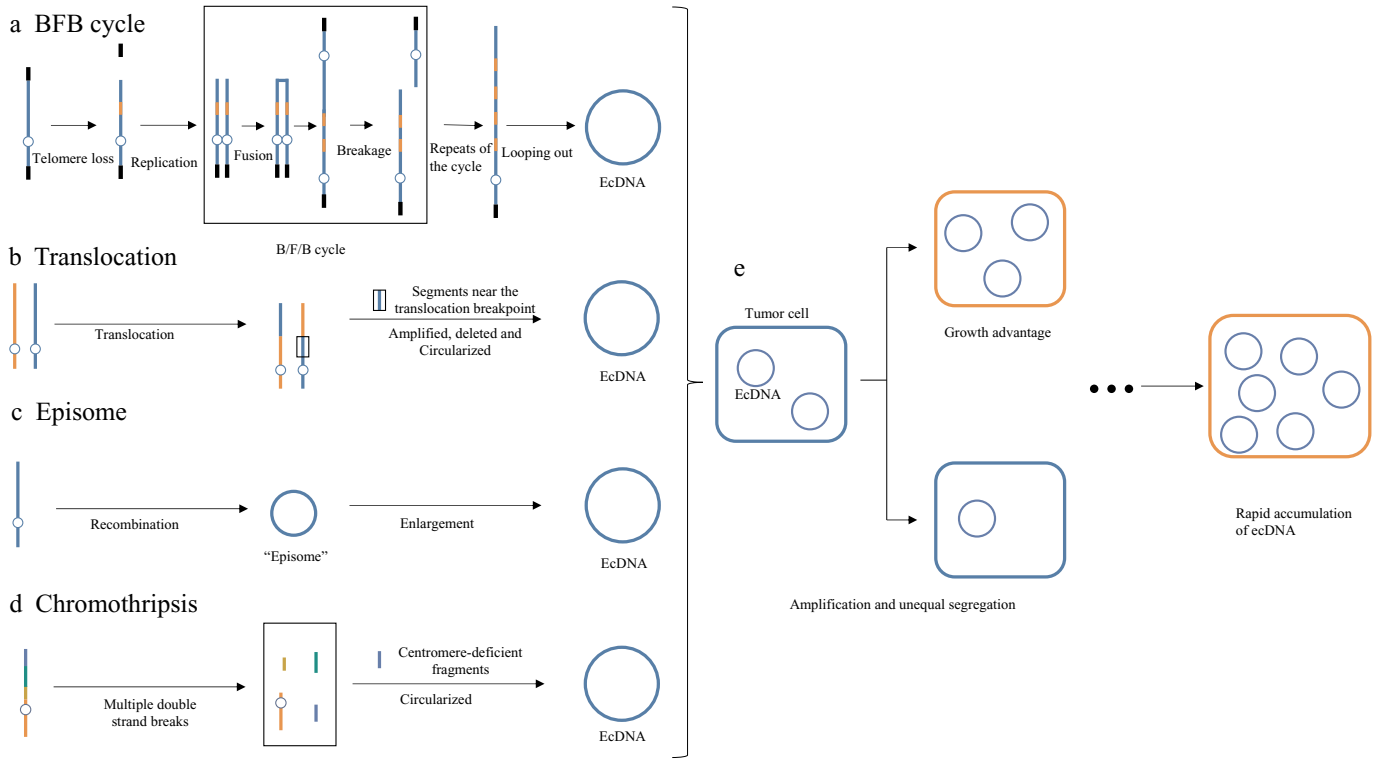

Fig. 1. Models of ecDNA formation and proliferation.
Four different models are proposed to illustrate the formation of ecDNA. a, The breakage-fusion-bridge (BFB) cycle is initiated by the fusion of broken chromosome ends and forms the dicentric anaphase bridge. The breakage of the bridge is random and uneven, and the subsequent products are segregated into daughter cells, one with duplication and another with deletion. Without a telomere on one side, the BFB cycle can be repeated. Eventually it may loop out and form ecDNA with gene amplification. b, The translocation-deletion-amplification model depicted the gene rearrangements that take place near the translocation site. The segments near the translocation breakpoints are either amplified, retained or deleted and therefore form ecDNA and HSRs. c, The "episome" model suggests that the episomes are autonomously replicating submicroscopic precursors of ecDNA. They are produced by a recombination event and can enlarge to form ecDNA. d, Chromothripsis is a catastrophic event in which a single or a few chromosomes are shattered into several double strand breaks. With the repair system, these fragments can be religated in random order causing complex locally clustered DNA rearrangements. In some cases, the fragments can ligate and circularize into extrachromosomal DNA elements such as EcDNA. e, During mitosis, ecDNA are amplified and unequally segregated into daughter cells, providing a pool of cells containing different copies of ecDNA. After several turns of mitosis and environmental selection, the cells with high copies of ecDNA elements may gain a competitive advantage.

containing high levels of these extrachromosomal elements may adapt better in some circumstances. Kristen used a mathematical model to predict the procedure and showed that the ecDNA amplification would increase the oncogene copy number far more effectively than the chromosomal way (like HSRs) [49]. They further validated that prediction by analyzing patient samples. In accordance with the high oncogene copies on ecDNA in cancer cells and the absence of ecDNA in normal cells, these results suggest that ecDNA plays a crucial role in tumor evolution.

5. The formation of ecDNA

The formation of ecDNA and HSRs has been long studied, and several models have been proposed, including the breakage-fusion-bridge (BFB) cycle, translocation-deletion-amplification model, "episome" model and chromothripsis (Fig. 1).

5.1. Breakage-fusion-bridge (BFB) cycle

One of the most classic models is the BFB cycle, which was first proposed by McClintock [53]. The BFB cycle is initiated by the fusion of broken chromosome ends caused by double-stranded DNA breaks, which subsequently forms the dicentric anaphase bridge [76]. Due to the presence of two centromeres, the breakage of the bridge is divided into daughter cells with duplication and another with deletion. Then, the whole procedure of the BFB cycle can be repeated, causing additional duplications and deletions. Depending on the location and size of the breakage, high levels of gene amplification such as HSRs and looping out as ecDNA can be generated [77].

5.2. Translocation-deletion-amplification model

Barrs found a novel mechanism in which translocation and amplification events cooperate to generate potent oncogenic activity [54]. It was summarized as a translocation-deletion-amplification model [78]. In this model, gene rearrangements take place near the translocation site. The segments near the translocation breakpoints are either amplified, retained or deleted and therefore form ecDNA and HSRs. The coamplification of MYC and ATBF1 and HMGIC and MDMD2 in tumor cells is observed under this model [79,80].

5.3. "Episome" model

Another model called "episome" was presented by Wahl and his colleagues. They found that gene amplification can be mediated by small circular extrachromosomal molecules, and referred to those molecules as "episomes" [55]. They suggested that the episomes are autonomously replicating submicroscopic precursors of ecDNA and are produced by a recombination event that deletes sequences containing a replication origin along with adjacent genes. The episomes can enlarge to form ecDNA or later integrate into chromosomes forming HSRs [81]. Evidence of the formation of MYC-containing ecDNA in leukemia,

neuroblastoma and small-cell lung carcinoma cell lines supported the episome model [82,83].

The formation of ecDNA and HSRs is always discussed under the same issue. With the highly identical structure of ecDNA and HSRs, evidence also shows that HSRs can evolve from ecDNA [66,83]. While HSRs are considered to be a stable form of gene amplification, ecDNA might act as a bridge between stable and unstable. The underlying mechanism for this change needs further investigation.

5.4. Chromothripsis

Different from these stepwise models, recent research shows a one-off event, named chromothripsis, resulting in genomic rearrangement in cancer [56]. It is a catastrophic event in which a single or a few chromosomes are shattered, forming tens to hundreds of sequence fragments. With the repair system, these fragments can be religated in a random order causing complex locally clustered DNA rearrangements [84]. Evidence of chromothripsis has been found in several tumor cells at high frequency [85–89]. In some cases, the fragments can ligate and circularize into extrachromosomal DNA elements [90]. EcDNA is detected associated with chromothripsis and results in oncogene amplification of MYC in small-cell lung cancer [56].

These models indicate multiple routes for ecDNA formation. Though the detailed steps need further investigation, a brief procedure was proposed [51]. First, the DNA fragments generate after the chromosome breakage, and some of them circularize to form ecDNA. Then, in the absence of tumor suppressors, these abnormal elements accumulate. Finally, those tumor cells with specific gene amplification in ecDNA fit the environment better and carry a high oncogene copy number.

6. The elimination of ecDNA and micronuclei

While the increased copy number of the oncogene on ecDNA promotes tumor heterogeneity, the elimination of the ecDNA amplified genes may reverse the phenotype. Treatment of dimethyl sulfoxide, ionizing radiation and hydroxyurea (HU) has been reported to reduce ecDNA in tumor cells [91–94]. Intriguingly, an increased frequency of ecDNA-contained micronuclei was observed following the reduction of ecDNA [95]. Micronuclei are a type of anomaly possibly induced from the acentric chromosome or chromatid fragments and enclosed by the nuclear membrane [96].

Thus, an important step involved in the loss of ecDNA is the entrapment of ecDNA within micronuclei [94]. The entrapment of ecDNA containing N-myc amplification was observed in vivo in neuroblastoma [95]. Shimizu depicted the possible procedure of the entrapment [97]. First, ecDNA sequences are primarily located at the nuclear periphery in the G Phase. Then, due to the DNA damage, ecDNA gets aggregated at S phase. Subsequently, ecDNA are incorporated into nuclear buds and removed from the nucleus through the formation of micronuclei. The nuclear budding process is considered to be a specific way for micronuclei formation [98]. However, after the entrapment, how the micronuclei content is further eliminated remains unclear. Several hypotheses are suggested for that, including that a replication defect may exist inside the micronuclei, the micronuclei are degraded in situ or they are extruded from the cells as extracellular micronuclei [99].

Another view on nuclear budding is suggested based on the observation of human colon carcinoma cells treated with nanomolar concentrations of doxorubicin. The formation of micronuclei was thought to be a mechanism for the segregation and elimination of redundant DNA or for generating viable aneuploid cells with a potentially extended life span [100]. Therefore, the elimination of ecDNA through micronuclei might be a key part of tumor progression under certain drug pressure.

7. Recent advances in ecDNA

To fully understand the comprehensive functions of ecDNA, more attention is paid to the dimensional and topological structure and the total sequences on ecDNA.

DNA and histones are compacted into complex hierarchical structures named chromatin, which restricts its accessibility to transcription [101]. Using assays for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) and micrococcal nuclease digestion and sequencing (MNase-seq), Wu et al. mapped the nucleosome position on ecDNA and found the high accessibility of chromatin residing on ecDNA [50]. They also illustrated the ultra-long-range chromatin contacts in ecDNA with the help of proximity ligation-assisted CHIP-seq (PLAC-seq) and circular chromosome conformation capture combined with high-throughput sequencing (4C-seq). This evidence shows that ecDNA promotes not only tumor pathogenesis through its high oncogene copy numbers, but also the alteration of chromatin structure.

With whole-genome sequencing and circle sequencing, Koche and his colleagues provide a genome-wide map of circular DNA in neuroblastoma [6]. Apart from the high frequency of known ecDNA and other small eccDNA, many previously undescribed extrachromosomal circular DNA were observed. They also provided evidence supporting that eccDNA contributes to cancer genome remodeling by forming chimeric circles and chromosomal circle integrations. Additionally, these circle-derived rearrangements are related to adverse clinical outcomes. Therefore, the circle-derived rearrangements may be another anticipated aspect of eccDNA related to tumor pathogenesis.

While past research focused mainly on the oncogene amplification itself, Morton and his colleagues set their sights on the amplified non-coding regions on ecDNA. They found that some neighboring enhancers are co-amplified with the oncogenes in the linear chromosome (EGFR in GBM and five more solid tumors) [102]. The co-amplification of enhancers was also seen in the circulation of these amplicons, the ecDNA. The specific circular topological structure provides new regulatory elements and new contacts on oncogenes. Morton suggests that these new connections based on the regulatory rewiring can provide a selective advantage that shapes extrachromosomal oncogene amplification.

8. Conclusions, discussion and perspective

The relationship between eccDNA and tumor has been discussed for decades. By virtue of new technologies such as next-generation sequencing and super-resolution microscopy, the latest findings on ecDNA in cancer do renew our interest. We summarized the possible biogenesis of different eccDNA elements with a focus on ecDNA. Several models have been proposed to explain the formation and proliferation of ecDNA, but the underlying mechanisms and direct evidence for each model still need further research, especially how these fragments of linear chromosomes ligate into circular form. In addition, direct evidence about whether ecDNA itself could replicate is missing. Shimizu et al. found that the occurrence of BrdU-labeled ecDNA is earlier than the BrdU-labeled HSR but later than BrdU-labeled chromosomal DNA [58]. Therefore, their data cannot exclude the possibility that the ecDNA amplification observed by BrdU labeling is directly from replicated chromosomes but not from its own replication. We propose that this question could be resolved by gene editing tools such as CRISPR-dCas9, which might be applied to visualize and track the dynamics of ecDNA in live cells [103].

In addition to tumor cells, circular DNAs can be found in many organisms in nature. For example, hepatitis B virus (HBV) replication induces the formation of covalently closed circular DNA (cccDNA), which contributes to the virus chronic infection in the human liver [104]. Recently, DNA lagging strand-synthesis machinery proteins were revealed to play an essential part in hepatitis B virus (HBV) relaxed-circular-DNA repair to form cccDNA [105]. These findings might

provide new insight into the biogenesis of eccDNA.

While the oncogene amplification on ecDNA promotes tumor pathogenesis, it is not a new idea to use ecDNA as a therapeutic target for treatment. We propose that the concomitant inhibition of ecDNA formation might enhance the effect of traditional antitumor drugs as a new strategy.

The frequency of ecDNA varies a lot in different cancers. While neuro tumors such as GBM can exhibit a high ecDNA-positive rate, others such as colon and hematologic cancer are usually lack of ecDNA. Additionally, we found that some pancreatic adenocarcinoma cells such as PANC-1, MiaPaca-2, AsPc-1 and SW1990 lack ecDNA (data not shown) by DAPI staining. Therefore, although ecDNA is considered to be a crucial part of tumor heterogeneity and progression, the significance of ecDNA may be dependent on specific cancer types.

The findings of nuclear budding and micronucleation also provide a possible way for ecDNA to escape from the cells. Similar to microDNA which is found in the plasma of pregnant women [106] and has been identified as a possible liquid biopsy biomarker for ovarian and lung cancer [41], ecDNA may release from tumor cells and serve as circulating tumor DNA (ctDNA) in patient serum.

In addition, there are still various undescribed eccDNA in tumor cells, and new elements may also be found with the cutting-edge tools. Overall, these new findings of eccDNA at both the basic and clinical levels bring us new views on cancer biology and may eventually result in the advancement of cancer diagnosis and treatment.

Authors' contributions

WJ conceived the manuscript. ZL collected the related papers and wrote the draft manuscript. ZJ, WJ, LY and TL revised the manuscript. XY and LL approved this manuscript. All authors read and approved the final manuscript.

Submission declaration

This manuscript and its contents have not been published or submitted for publication elsewhere. All authors have contributed significantly, are in agreement with the content of the manuscript, and have agreed its submission.

Declaration of Competing Interest

Authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from the National Science Foundation for Distinguished Young Scholars of China (81625016), the National Natural Science Foundation of China (81871941, 81872366, 81827807, 81802675, 81701630 and 81702341), the Outstanding Academic Leader Program of the “Technological Innovation Action Plan” in Shanghai Science and Technology Commission (18XD1401200), the Scientific Innovation Project of Shanghai Education Committee (2019-01-07-00-07-E00057), the Natural Science Foundation of Shanghai (19ZR1410800), Clinical and Scientific Innovation Project of Shanghai Hospital Development Center (SHDC12018109), and the Young Talented Specialist Training Program of Shanghai.

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