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Review Article

Extrachromosomal DNA (ecDNA): an origin of tumor heterogeneity, genomic remodeling, and drug resistance

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The genome of cancer cells contains circular extrachromosomal DNA (ecDNA) elements not found in normal cells. Analysis of clinical samples reveal they are common in most cancers and their presence indicates poor prognosis. They often contain enhancers and driver oncogenes that are highly expressed. The circular ecDNA topology leads to an open chromatin conformation and generates new gene regulatory interactions, including with distal enhancers. The absence of centromeres leads to random distribution of ecDNAs during cell division and genes encoded on them are transmitted in a non-mendelian manner. ecDNA can integrate into and exit from chromosomal DNA. The numbers of specific ecDNAs can change in response to treatment. This dynamic ability to remodel the cancer genome challenges long-standing fundamentals, providing new insights into tumor heterogeneity, cancer genome remodeling, and drug resistance.

Introduction

Many oncogenes that were thought to reside only on linear chromosomes have now been shown to be present in large circular extrachromosomal DNA (ecDNA) (Figure 1, Table 1). First documented in the 1960s as double minute chromatin bodies [1] and then elegantly studied in the late 1970s and early 1980s [2–6] these extrachromosomal DNA structures were of keen interest, but were overshadowed by advances in molecular biology and powerful genomic technologies that permitted more exquisitely detailed mapping of genomes, including tumor genomes. However, this increase in genome resolution came at a cost of spatial resolution, because the DNA sequence reads were mapped back to the chromosomal genome map derived from normal cells (see Box 1). Therefore, something important was overlooked: the ecDNAs of cancer cells. The straightforward use of fluorescence *in situ* hybridization (FISH) probes to examine the location of amplified oncogenes in cancer cells was a milestone for cancer research as it brought ecDNA into the light again [7].

ecDNA was thought, until very recently, to be a rare characteristic of tumors (1.4% of tumors according the Mittelman database of chromosomal aberrations), of unclear significance but current evidence shows that highly amplified, oncogene-containing ecDNAs are common in cancer (25 out of 29 types) [8]. They are a cancer-specific subset of the collective term, extrachromosomal circular DNA (eccDNAs) which include various types and sizes [9,10] and differ from the small eccDNAs that are found in normal human cells, such as muscle and leukocytes [11] (Figure 2). Cancer cell ecDNAs are often amplified to a high level, are large mega-base pair structures (>1 Mb), and contain many genes and regulatory regions. In contrast, eccDNAs found in normal cells, are not amplified, are small, (usually <3 Kb), and usually do not encode proteins. Furthermore, ecDNA have to date, not been found in normal human cells or tissues [8].

Key Figure: Dimensions of ecDNA

Formation *via*
Chromothripsis,
areas of high transcription,
aborted apoptosis,
DNA damage

Structure:
Circular shape, large size,
lack of centromere,
abundance in cancer,
and composition from
chromosomal origins

Expression-
Open chromatin,
novel cis- and trans-regulation,
transcriptional hubs

?

Oncogene activation via
amplification and
Tumor suppressor inhibition via
integration into host chromosomes

ecDNA

Therapy and Biomarkers:
- Enzymatic targets
- Fusion sites as a source of neoantigens
- Vehicle for TS genes

?

Tumor heterogeneity and tumor evolution

Genomic remodelling

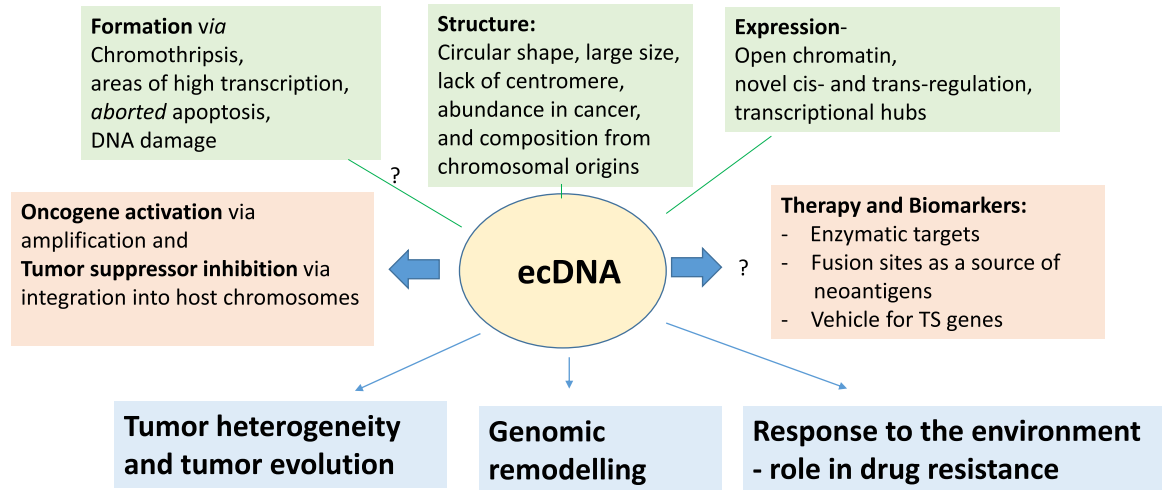**Response to the environment - role in drug resistance**

Figure 1. Dimensions of circular extrachromosomal DNA (ecDNA) in tumor cells.
The basic physical and functional characteristics of ecDNA are shown in green text boxes. These underlie three important dynamic processes shown in blue text boxes. The consequences of these processes can lead to oncogene activation and/or tumor suppression and may also lead to new therapies and/or biomarkers indicated by large arrows. Possible mechanisms of formation and therapeutic strategies that require further investigation are marked by a '?'.

The physical structure of the ecDNAs has been mapped and has been found to have a great impact on function. First, ecDNAs lack centromeres and do not follow the rules of Mendelian inheritance, driving intra-tumoral heterogeneity. Second, the circular topology of ecDNA is characterized by highly accessible chromatin (see Box 2), a paucity of repressive histone marks with a high level of active histone marks, affecting the epigenomic and transcriptional landscape [12]. There is clearly elevated oncogene expression, even when normalized for copy number. Lastly, the circular architecture creates new *cis*-regulatory interactions as regulatory elements that are too far away to interact on a linear chromosome are brought into proximity on a circle [13]. Furthermore, ecDNAs have now been shown to engage in intermolecular interactions, between ecDNA particles [14,15] and between ecDNA particles and chromosomes [16]. ecDNAs can also reintegrate into chromosomal regions in non-native loci (sometimes referred to as homogenous staining regions (HSRs)), resulting in enhancer hijacking [17].

Table 1. Commonly amplified oncogenes and frequency on ecDNAs in tumor samples [8]

Gene name	Percentage on ecDNA
CDK4	62.1
MDM2	59.7
AKT1	47.1
E2F3	40.7
NEDD9	39.5
EGFR	39.1
MYCL	38.1
PDGFRA	37.5
SOX2	36.4
TERT	32.9
MYC	26.6
ERBB2	25.5

ALL nuclear extrachromosomal circular DNAs of endogenous chromosomal origin
(eccDNAs)

Healthy cells   Cancer cells

Small (Kb size) eccDNAs
- not amplified
- does not usually contain
  whole genes

Large (Mb size) cancer cell-specific = ecDNAs
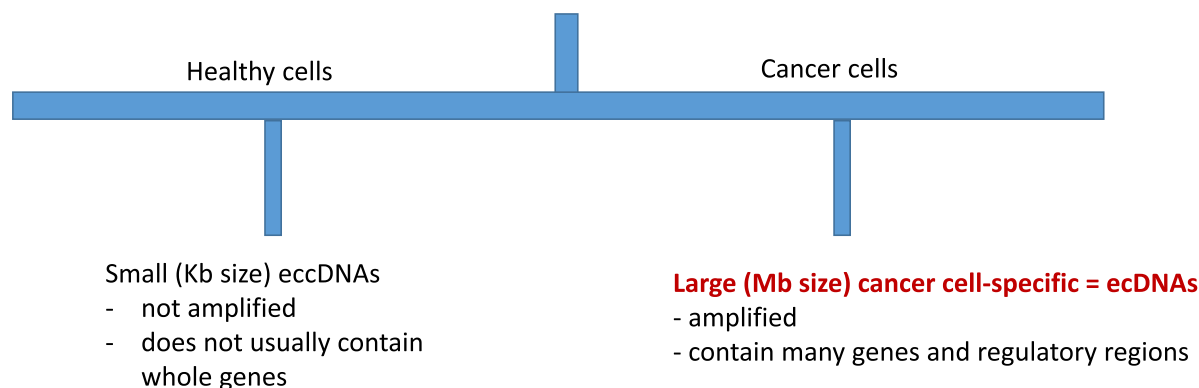- amplified
- contain many genes and regulatory regions

Figure 2. Extrachromosomal circular DNA (eccDNA) is a collective term that includes various types and sizes derived from chromosomal origins.
Large (>1 Mb) cancer-specific circular extrachromosomal DNA (ecDNA; red text) is a subset of eccDNAs that often carry oncogenes [9,10]. This paper focuses on ecDNAs.

BOX 1. Cancer Cartographers
Cancer biologists are modern day cartographers, creating visual representations to navigate altered tumor genomes. These cancer genomic maps have helped reshape the collective understanding of cancer pathogenesis and are being used to guide precision treatment. However, recent work suggests that for some cancers, the maps are misleading, despite being made from accurate and precise genomic measurements. This idea of right measurements - wrong maps, is not new and is not unique to cancer. For example, in ancient times, the astronomer Ptolemy made precise measurements of the planets moving across the night sky. The measurements were good, but the map was wrong because he placed the Earth in the center. It took nearly 1400 years for the map to be revised by Copernicus, based on the same measurements. By placing the Sun in the center, the map gained new explanatory power and fostered a new scientific revolution. Thus, remapping can have profound implications. The same applies for genomic maps of cancer.

BOX 2. Mapping of ecDNA topology
Accessible chromatin can be mapped using the assay for transposase-accessible chromatin (ATAC) and visualized (ATAC-see). A transposase enzyme is used to label open chromatin structure that lack tightly packed nucleosomes with a fluorescent tag.

Uneven segregation and clustering of ecDNA elements fuel tumor heterogeneity

During mitosis, replicated DNA is segregated equally to create identical copies of the genome and normally results in identical daughter cells. Microtubule spindle structures attach to the kinetochore complex at the centromere and direct chromosome alignment and segregation. Centromeres or replication origins have not been detected on ecDNA elements, implicating an absence of the kinetochore complexes that dictate the organization of the mitotic spindle. ecDNAs are therefore predicted to be randomly distributed across the daughter

cells during mitosis [18]. As a consequence, ecDNA elements are inherited in a radically different fashion in comparison with chromosomes.

Two recent studies, using live cell imaging and tracking and image analysis to count ecDNA distribution after cell division, extend *in situ* single point observations over time, and demonstrate random ecDNA segregation during mitosis [15,19]. Uneven segregation creates an imbalance in the number of ecDNA elements that gets distributed between daughter cells and results in an approximate Gaussian distribution in the per-cell content of DNA after mitosis. These findings are consistent with the distribution of ecDNAs among cells in clinical samples [8,20]. This random distribution results in an increase in gene copy number in a subset of cells leading to tumor heterogeneity and represents another mechanism of gene amplification in addition to that which occurs on linear chromosomes.

Crucially important, this uneven segregation may lead to accelerated evolution of cancer cells. Tumor cells containing ecDNAs that are enriched for growth-promoting oncogenes and/or pro-growth regulatory elements and not other types of ecDNAs, may outcompete other cells. This is reminiscent of the consequence of plasmids in bacteria. Circular DNA that is transmitted randomly to daughter cells favors rapid change. ecDNAs have recently been shown to mark the clonal expansion that takes place in newly cultured patient-derived glioblastoma (GBM) cells and orthotopic xenografts of cultured GBM compared with their disappearance in cultured neurospheres, where clonal selection is absent [21].

ecDNA composition, dynamics and its consequence for genome integrity

To shed light on the composition of ecDNAs, the cancer biologist must return to mapping at nucleotide resolution using specific sequencing techniques. Whole genome sequencing and the use of bioinformatics can determine the nucleotide sequence of ecDNA relative to a reference genome as a region of increased copy number [22]. Features such as soft-clipped reads (portions of the read that do not match well to the reference genome) help to identifiy circle junctions. The read length is limited to ∼200 bp and so long-read sequencing is required to resolve complex ecDNA rearrangements. Another tool is Circle-Seq, which enriches for ecDNA by using an exonuclease to remove linear DNA and is not dependent on ecDNA copy number [23]. Applying Circle-Seq to ecDNAs reveals a remarkable diversity that cannot be detected even by single cell DNA or RNA sequencing. These diverse ecDNAs within a single cell can be classified into at least two types. One type is composed of simple single fragment DNAs, containing an oncogene and local enhancers that drive oncogene expression (Class 1). Alternatively, they can be chimeric, containing multiple fragments from several different chromosomes (Class II). In addition, it has been proposed that some ecDNAs may not contain any oncogenes but rather only contain enhancers (Class III), which may drive the expression of oncogenes on other ecDNAs in ecDNA hubs (see below) and utilize their enhancers in trans. These characteristics suggest an intriguing possibility that intracellular ecDNA diversity and competition may potentially drive tumor evolution.

A new addition to methods for analysis of ecDNA containing cells is CRISPR-CATCH, which uses *in vitro* CRISPR–Cas9 treatment of agarose-entrapped genomic DNA followed by pulse field gel electrophoresis. In brief, an agarose solution is added to cancer cells to create an agarose plug containing intact genomic DNA. CRISPR–Cas9 can be directed by sgRNAs to produce a single cut within the target gene on ecDNA and a double cut on the boundaries of the target chromosomal locus. The products are separated by pulse field gel electrophoresis and can be extracted for subsequent sequence analysis. One advantage of this approach is that genetic and epigenetic variations of both chromosomal and ecDNA sequences in one sample can be compared. It has been demonstrated in one analysis that an EGFRvIII mutation was present on ecDNA while the wild-type EGFR gene was located on chromosomal DNA and the promoter of this target gene was hypomethylated in ecDNA compared with the chromosomal locus [13]. It is anticipated that this approach will accelerate structural analysis of both ecDNAs and HSRs.

There are several proposed mechanisms for the formation of ecDNAs (Figure 1). Processes that involve DNA damage are important candidates. These may include double-strand breaks and breakage-fusion-bridge cycles. Chromothripsis, the shattering of a chromosomes, has been identified as a mechanism for some ecDNAs (∼36%) [8]. The formation of some ecDNAs may be associated with a deletion of the locus on the chromosome of origin, resulting in chromosomal scarring. The fusion of a single DNA fragment into a circle generates a novel tail-to-head fusion point. Similarly ecDNAs with DNA fragments from different chromosomes will have multiple breakpoints junctions. EcDNA-breakpoint specific guide RNAs in combination with a deactivated Cas9 protein can be used to tag ecDNAs in live cells, thus providing an opportunity to visualize
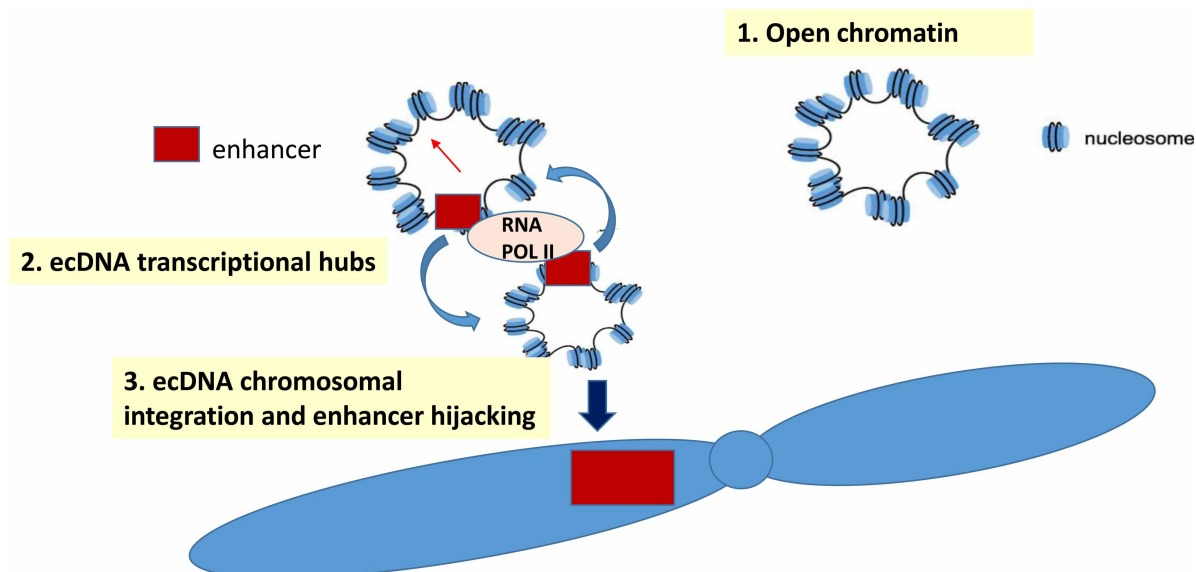
Figure 3. The epigenetic and transcriptional landscape.

1. Circular ecDNAs have an open chromatin structure. 2. ecDNAs aggregate in transcriptional hubs that allow for trans-regulation (blue arrows). Circular ecDNAs allow for novel cis-interactions (red arrow). RNA Polymerase II (RNA Pol II) has been identified at these hubs. 3. ecDNAs can integrate into chromosomes and create novel enhancer interactions.

dynamic ecDNA behavior. Evidence shows that ecDNAs do not randomly distribute within a cell, but rather form clusters, called ecDNA hubs, which promote intermolecular interactions and which overlap with RNA polymerase II [16] to drive transcription [14,15,24].

Hubs containing clusters of 10–100 ecDNAs have been shown to be tethered together by the bromodomain and extraterminal domain (BET) protein, BRD4, in a colorectal cancer cell line containing MYC ecDNA [14]. BET proteins are localized at super-enhancers that flank MYC and are involved in its regulation. A BET inhibitor disperses the hubs and inhibits ecDNA-oncogene expression. These investigators also mapped intermolecular enhancer-gene interactions by CRISPR interference and demonstrated that enhancers on some ecDNAs could activate genes on other ecDNAs. Gene expression is higher in ecDNA hubs compared with individual ecDNAs due to transactivation.

Furthermore, recent evidence for intermolecular ecDNA-chromosomal interactions between genes and regulatory elements dramatically increases the diversity of potentially important forms of transcriptional regulation of the cancer genome [13,16]. See Figure 3.

ecDNAs also have the potential to remodel the chromosomal genome of a cancer cell through reintegration into chromosomal regions that are not usually their native locus, creating HSRs. HSRs is a term used to describe the FISH staining pattern showing large collections of the amplicon on chromosomes. Recent analysis of chromosomal HSRs provide evidence of reintegration of ecDNAs at these sites [20,21]. Reintegration into chromosomes can have several mutational consequences including the *mis*-regulation of resident oncogenes due to relocated enhancers and the interruption of tumor suppressor genes, as demonstrated by Koche et al. [17]. They demonstrated that ecDNA integration sites were significantly enriched for cancer-relevant genes, especially tumor suppression genes. Analysis of one neuroblastoma genome indicated a chromosomal insertion that disrupted the tumor suppressor gene DCLK1. Gene expression was significantly decreased as expected. Clinically, poor prognosis is associated with low DCLK1 expression. On the other side of the coin, increased expression of the oncogene TERT was seen upon ecDNA insertion close to the regulatory region of this gene. Thus, ecDNAs appear to be agents of genomic rearrangements, which may lead to dysregulation of oncogenes and tumor suppressor genes.

The structure of ecDNAs themselves may be subject to ecDNA integration and other structural changes. Structural analysis identified a mirror-image repeat of a KRAS fragment suggesting that two ecDNA molecules had merged [12, extended data]. Evidence is also gathering for the evolution of ecDNA structure over the
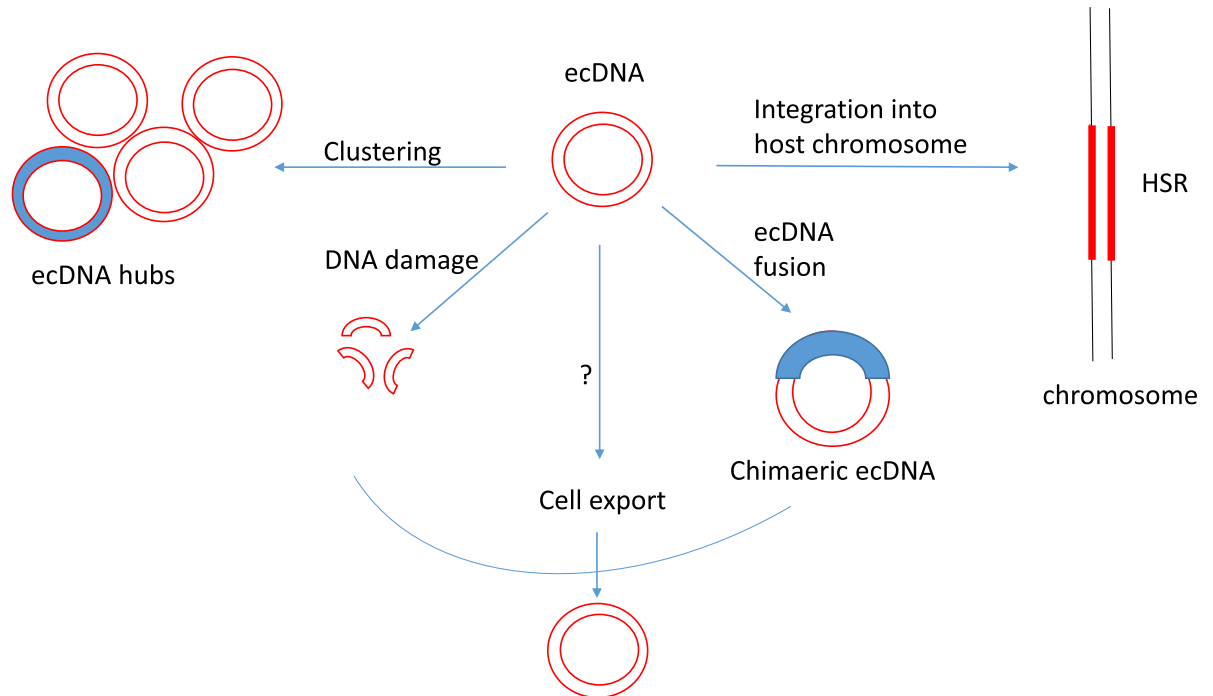
Figure 4. Fates of ecDNA.

Fates of ecDNA include clustering to form ecDNA hubs which enable *trans*-transcriptional regulation, integration into host chromosomes that may give rise to homologous staining regions (HSRs), loss due to DNA damage, fusion with other ecDNAs to create chimaeric ecDNA, and possibly [indicated by '?'] cell export.

course of cancer progression. Additional fates of ecDNA may include loss by DNA damage such as that caused by radiation, and cell export (Figure 4).

ecDNA induced drug resistance

The failure of many cancer treatments is due to the development of drug resistance. Drug resistance in cancer patients cannot simply by explained by time-consuming selection of drug-cancelling mutations. Others have linked tumor heterogeneity with drug resistance and/or proposed a reversible drug-tolerant state in individual cells of a tumor [25]. Cancers with ecDNAs appear to change their genomes at fast rates potentially explaining why patients whose cancers harbor ecDNA have shorter survival than other cancer patients, even when tumor type and plausible confounding factors is taken into account [8]. Rapid treatment resistance, driven by the remarkable genome plasticity engendered by ecDNA is likely to play a key role.

Several studies confirm alterations in the abundance of ecDNAs upon drug treatment [7,19,26]. Some drug treatments result in an increase in ecDNA copy number to develop drug resistance while other drug treatments lead to decline of ecDNAs carrying the drug target to develop drug resistance. This depends on the selection pressure needed for higher cell growth or fitness (Table 2). For example, methotrexate is a drug that targets an important enzyme of nucleotide metabolism called dihydrofolate reductase. Treatment of cells with methotrexate results in a rise in the number of ecDNAs that carry the dihydrofolate reductase gene (*DHFR*) leading to methotrexate resistance. In this case, an increase in the drug target leads to better cell fitness. In contrast, ecDNAs carrying a mutant of *EGFR*, (*EGFRvIII*) that increases the sensitivity of cells to EGFR inhibitors, decrease when cells become drug resistance. Staining of HSRs in these cells suggest that these ecDNAs reintegrated into chromosomes. This relocation of ecDNAs is reversible as mutant-carrying ecDNAs reappear upon removal of the drug. A direct comparison of effects after treatment with EGFR inhibitors between isogenic cell line pairs- one containing amplification of an EGFRvIII on chromosomal HSRs and the other containing this amplification on ecDNAs, showed the HSR cell line remained sensitive to drug while the ecDNA containing

Table 2. Effects of types of selection on ecDNA copy number

	Negative selection	Neutral selection	Positive selection
ecDNA target gene	*EGFRvIII* mutant	*DHFR*	*DHFR*
Treatment	EGFR inhibitor	none	methotrexate
ecDNA copy number	Decreases	Remains the same	Increases

cells became resistant in 2 weeks [19]. The implication of ecDNAs as a mechanism for drug resistance places it as a most important target for future therapy.

Clinical implications of ecDNAs

Since the first report of ecDNAs in neuroblastomas in 1965 [1], various reports highlight their importance in clinical contexts. In 1985, Seeger et al. [27] provided clinical evidence that amplification of the oncogene *MYCN* was associated with worst prognosis. Ever since, MYCN amplification was used routinely in clinical risk stratifications of patients suffering from neuroblastoma. Even though the presence of *MYCN* amplifications is associated with adverse outcome, great inter-individual outcome heterogeneity can be observed, which remains a conundrum in the field. More recently, ecDNA-derived chromosomal rearrangements involving MYCN was shown to be associated with worse overall survival compared with patients with MYCN amplifications without such chromosomal rearrangements. This suggests that circle-derived rearrangements may explain some of the clinical differences observed in MYCN-amplified neuroblastomas [17].

The reward of understanding the molecular players in cancer is to be able to use the information towards the development of new therapeutics.

Concluding remarks and future perspectives

The newly evaluated mapping and characteristics of ecDNA changes many fundamentals of what we know about cancer. First, ecDNAs provide an accelerated mechanism for heterogeneity, mutation, and the genomic evolution of a tumor. The proposal that this may impact the development of drug resistance may be crucially important for clinical translation. Secondly, gene regulation of oncogenes on ecDNAs are much more complex as a result of ecDNA structure and dynamics. This is due to the range of *trans*-regulation that is possible across different enhancers brought together on circular structures, the formation of ecDNA hubs, and the relocation and/or trans regulation of chromosomal enhancers.

More recently data suggests that *de novo* mutagenesis of ecDNAs may occur via APOBEC3, an enzyme that acts upon the circular genomes of pathogenic viruses such as papillomaviruses and polyomaviruses as part of an antiviral defence mechanism [28]. APOBEC3 is a cytidine deaminase that can lead to a specific pattern of localized hypermutation called kataegis. Thus, ecDNA may fuel carcinogenesis by its role as a novel target for mutagenesis by APOBEC3, in addition to its roles in oncogene expression and amplification and chromosomal rearrangements.

Currently, the mechanisms that drive ecDNA formation are not well understood. Chromothripsis provides one plausible mechanism that has been elegantly demonstrated [29,30], the 'fingerprints' of which have been found in a little over a third of ecDNA-containing cancers [8]. Other mechanisms, including paired double-strand breaks, breakage-fusion-bridge cycels and transcription-replication collisions may also be implicated. It is fascinating to note lessons from lower model organisms such as yeast, which routinely amplify environmental resistance genes on circular extrachromosomal DNAs, a mechanism that has been postulated to be transcription-induced [31]. DNA supercoiling that occurs during transcription is resolved by topoisomerases. Topoisomerase II can lead to double-strand breaks and it is in the repair of this damage that ecDNAs may be formed. This may help answer the question of why oncogenes are often found on ecDNAs. The overlap of ecDNAs and RNA Polymerase noted above may also imply high topoisomerase II activity that can create double-strand breaks as a mechanism for reintegration into linear chromosome or other ecDNAs. Future studies will be needed to dissect the mechanisms of ecDNA formation and maintenance, and assess their actionability.

As a tumor-specific molecular feature, ecDNAs become obvious potential diagnostic biomarkers and therapeutic targets.The first steps towards investigating their use as diagnostics has been taken by the identification of eccDNAs in circulating cell-free DNA (cfDNA) [32] and the identification of eccDNAs (<2 kb) in urine [33]. Although ecDNA sized particles were not reported, these studies demonstrate that circular DNA is released into the circulation. The closed circular topology of eccDNAs make them less susceptible to exonucleases compared with linear chromosomal cfDNAs. Further studies, such as those examing eccDNAs as biomarkers in lung adenocarcinoma [34], are needed to evaluate their sensitivity and specificity as cancer diagnostics. Less is known about potential therapeutic targets of ecDNA. It may be postulated that therapeutic targets may include enzymatic activities involved in ecDNA formation or interference of amplified oncogene expression that occurs from clustering in ecDNA hubs-proof of principle demonstrated by BET inhibitors (discussed above). Novel circle junction sequences in ecDNAs may also act as drug targets. It is also possible to envisage that synthetic ecDNAs can be used to deliver tumor suppressor activities or therapeutic agents.

It may be worth exploring whether common neoantigens are produced from novel sequence ecDNA expression and whether patients exhibit antibody reactivity against them. If this was the case, they could facilitate the development of cancer vaccines in a similar way that frameshift peptides are being investigated for cancer vaccines [35]. Our current knowledge on the relationship of ecDNA with the immune system is sparce although some data suggest that ecDNA formation leads to evasion of the immune system [36] and a blunted immune response. Further investigations will create another avenue of research that may lead to new immunotherapies.

And lastly, if ecDNA is a critical player in drug resistance, can it be targeted to stop quick tumor evolution and adaptation and increase drug response?

Cancer biologists are once again cartographers with a new map in hand. It is an exciting time and one in which insights into the role of ecDNA, long anticipated, can now be more fully realized by an expanded tool kit, potentially translating this emerging hallmark of cancer [37] into better treatments for cancer patients.

Perspectives

- The cancer genome is NOT static; it is dynamic.

- The application of current molecular analysis to ecDNAs is a new endeavor and is uncovering new fundamentals about the remodeling and evolution of the cancer genome.

- ecDNAs underlie characteristics such as tumor heterogeneity and drug resistance and new mechanisms of carcinogenesis through altered oncogene and tumor suppressor expression.

- ecDNA contributes to the three pillars of Darwinian evolution (inheritance, variation, and selection) in ways that differ from contributions from linear chromosomes.

- As a common molecular marker, not found in healthy cells, ecDNA and associated partners may become important diagnostic and drug targets and help us to unravel drug resistance, the crux of cancer treatments.

Glossary

breakage-fusion-bridge cycles a cycle of telomere breaks and dicentric chromosome formation that leads to chromosomal instability and possibly ecDNA formation.

ecDNA a distinct type of DNA that does not reside on a chromosome, is circular, and is commonly observed to carry oncogenes in human cancer cells.

ecDNA hubs clusters of ecDNAs (10–100) that form in the nucleus and may facilitate trans-ecDNA gene expression

enhancer hijacking the use of a distal enhancer that has been translocated. In the context of ecDNA, this may occur by DNA circularization, ecDNA hub formation, or reintegration into chromosomes.

Clonal expansion the process by which daughter cells arise from a parent cell

Clonal selection the concept that those cells that can respond to a changing environment (due to genomic changes) will proliferate and survive.

Guide RNA a fragment of RNA used to target specific RNA or DNA sequences with genome editing enzymes such as Cas9

Homogenous staining regions (HSRs) a pattern of extensive fluorescent signal seen on chromosomes after analysis by Fluorescent *in situ* hybridization often seen in cancer cells. It indicates a region of DNA sequence amplification and can be marker of ecDNA reintegration.

Competing Interests

Acknowledgements

Abbreviations

ATAC, assay for transposase-accessible chromatin; BET, bromodomain and extraterminal domain; DHFR, dihydrofolate reductase gene; GBM, glioblastoma; HSRs, homogenous staining regions; ecDNA, extrachromosomal DNA.

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