Tethering of ecDNA to Chromosomes during Mitosis in Cancer Cells

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Abstract

Extrachromosomal DNA (ecDNA) emerges as a result of DNA amplification. It is present in many diverse cancer types leading to therapy resistance and poor patient outcome. The random inheritance of ecDNA leads to enhanced tumour heterogeneity. The aim of our study was to get closer to understanding the mechanism underlining tethering of ecDNA during cell division. In order to reach that objective, we proceeded with fluorescent in situ hybridization (FISH), immunofluorescence staining (IF) and immunofluorescent staining in situ hybridization (IF-FISH) protocols on samples of cervical cancer cell line (HeLa) with ecDNA treated with various concentrations of Caseine Kinase Inhibitor (CX4945), which affects interaction of TOPBP1 and MDC1 proteins. It was shown that they are involved in tethering acentric fragments of chromosomes that had DNA damage, therefore we thought they might have a role in tethering other acentric fragments such as ecDNA. Using the drug CX4945 allowed us to assess the role of TOPBP1 and MDC1 in ecDNA inheritance. We are suggesting that TOPBP1 and MDC1 are vital for tethering of ecDNA to chromosomes. Nevertheless, the specific mechanism is still unclear and needs to be further researched.

Introduction

DNA amplification is the process in which a DNA region copy number is increased (1). The amplification of DNA regions can contain up to hundreds of copies (2). EcDNA is a type of DNA amplification and is a common occurrence in various cancer types. It contributes to unfavourable patient outcomes due to the frequent development of therapy resistance and accelerated cancer evolution. The production of ecDNA can occur through mainly two different mechanisms. Firstly, through double-strand breaks (DSB) followed by end-to-end ligation. Secondly, through the process of chromothripsis, where chromosomes undergo shattering

and reassembly. Moreover, ecDNA can evolve either through acquiring more genetic material, fusion of multiple ecDNA or through reintegration into the linear genome (2).

To comprehend the segregation of ecDNA in cells during mitosis, it is imperative to understand how cell proliferation functions. Mitosis is one of the most pivotal points in any cell, orchestrating the division of a parent cell into two genetically identical daughter cells (figure 1). Prior to mitosis each chromosome creates an exact duplicate of itself. The chromosomes condense and coil generating the classical X-shape with a centromere tying the two chromatids together. During metaphase the chromosomes align along the central plain, ready to be divided equally to each side of the nucleus. The centromeres play a vital role in the anaphase, as microtubules split the chromosomes equally at the centromeres, pulling each half to an opposing pole. As telophase progresses the nuclear membrane forms, splitting the cell into two equal daughter cells (3).

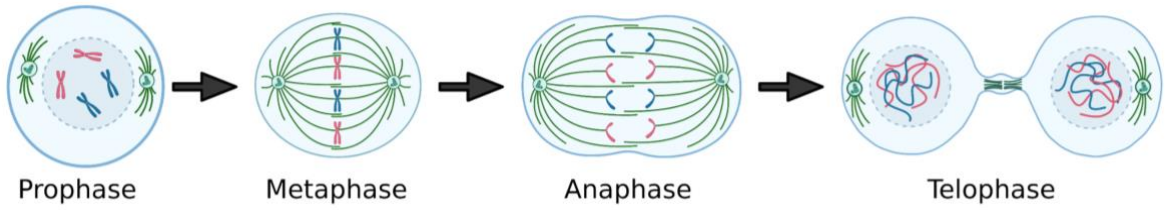

Figure 1: Representation of all phases of mitosis

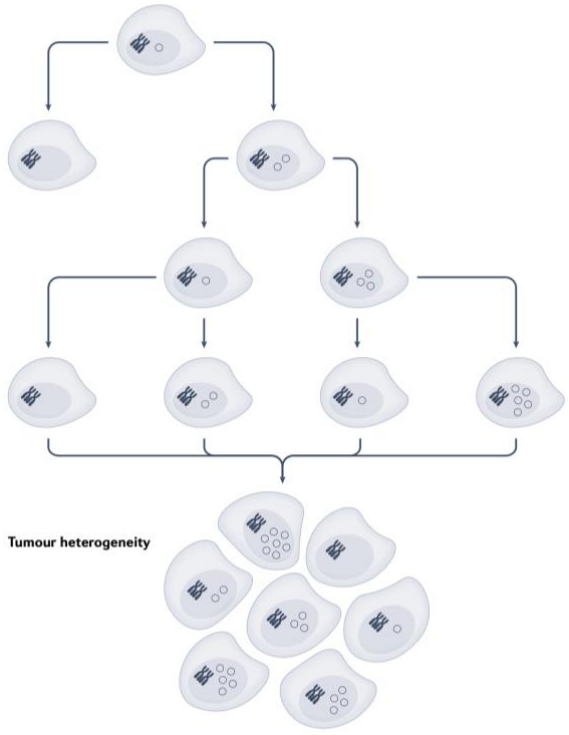

Figure 2: ecDNA's effect on tumor heterogeneity (2)

In contrast to chromosomes, ecDNAs do not contain centromeres, thus they cannot be segregated equally during mitosis, resulting in tumour heterogeneity (figure 2) (2). Heterogeneity in cells is harmful because it limits the effectiveness of cancer therapies (4). Researchers observed that segregation of ecDNA occurs due to tethering between ecDNA and chromosomes. However, the mechanisms underlying the tethering are unclear and will be explored in this paper.

A possible explanation involves MDC1-TOPBP1 complex, which is responsible in facilitating tethering of broken ends of chromosomes following a DSB. Additionally, MDC1-TOPBP1 has been shown to be involved in tethering of DSB during mitosis (5).

Consequently, we hypothesize that the MDC1-TOPBP1 complex might play a role in tethering acentric and fragmented DNA such as ecDNA (6). In our experiment we use a drug called CX4945 which disrupts the interaction between the MDC1-TOPBP1 complex. Thus, if the MDC1-TOPBP1 complex indeed is responsible for tethering of ecDNA and the chromosomes, there should be low or even no tethering seen when the drug is applied.

Material and Methods

The methods we used for detecting whether TOPB1 and MDC1 are involved in the tethering of ecDNA to chromosomes are FISH, IF and IF-FISH. Starting off with the mechanisms of FISH, a DNA probe is labelled with fluorophore using DAPI staining which is used for counting the number of nuclei. This is followed by the denaturation of the probe and target DNA at high temperatures resulting in DNA splitting into single strands. The annealing phase follows- the DNA and the probe can bind due to the complementary sequences. The fluorescent probe can then be detected in the chromosome which in turn reveals the presence and location of the target DNA (9). FISH has numerous uses such as monitoring effects of therapy and identifying chromosomal abnormalities, and what we used it for in our project, detecting tethering of ecDNA on the chromosomes (10).

The second method we used was IF which is a technique used for the development of the immune fluorescent cells (11). The protocol incorporates cultivation, fixation, staining and imaging, which also includes primary and secondary antibody incubation (12). The purpose of the primary antibody is to detect and bind to the target molecule due to its specificity to the molecule, whereas the secondary antibody is responsible for binding to the primary antibody as it carries the fluorophore (13). Our last protocol IF-FISH includes a combination of both methods.

Our samples were cells containing ecDNA (with DHFR[1]). The concentrations of CX4945 that we used were 0µM (control), 5µM and 10µM. Further in our discussion we divided the results of CX4945 on tethering of ecDNA into ‘100%’ (all ecDNA seen on or very close to the chromosome), ‘high’ (most ecDNA seen on or very close to the chromosome with some ecDNA seen around the chromosome) and ‘low’ tethering (most ecDNA seen around the chromosome).

Once all our samples were ready, we used the Echo and Nikon spinning disc confocal microscopes to analyse our results for high accuracy. We looked for mitotic cells that possibly had ecDNA tethered to the chromosomes along with the MDC1 and TOPBP1 proteins.

[1] DHFR is an enzyme that is crucial for DNA synthesis

Results

Firstly, we observed tethering of ecDNA and chromosomes in mitotic cells (figure 3). Additionally, the microtubules were stained in purple. We added this figure in order to display the tethering of ecDNA on chromosomes during the process of mitosis.

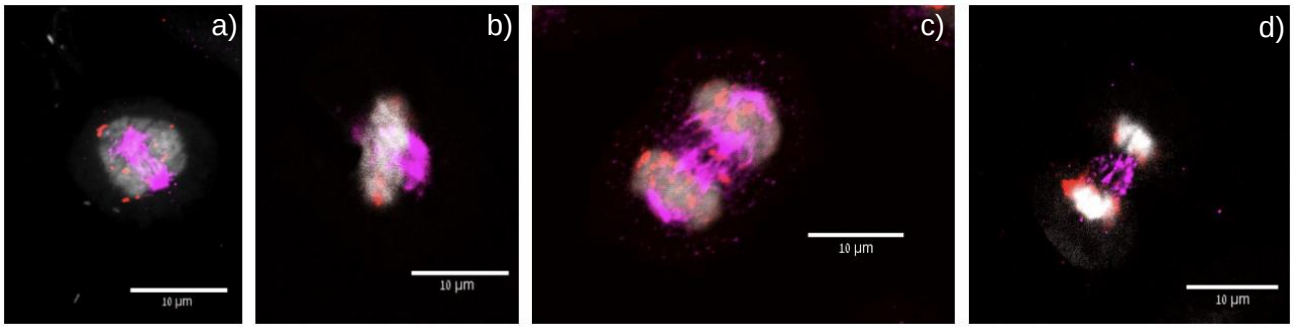

Figure 3: Visual representation of tethering during mitosis of ecDNA. Starting from left, the pictures show prophase (3a), metaphase (3b), anaphase (3c), and telophase (3d). Purple represents microtubules, white represents chromosomes and red represents ecDNA

Furthermore, we looked at examples of tethering of ecDNA to the chromosomes and the co-localization of ecDNA, MDC1 and TOPBP1 in some of the control samples (figure 4 and 5). Figure 4 is an example of '100% tethering', as all ecDNA is either on or very close to the chromosomes. In contrary, in figure 5 there is 'high tethering' because most of the ecDNA is present on the chromosomes, with some ecDNA around the chromosomes in figure 5. Moreover, the co-localization of ecDNA and TOPBP1 is shown (figure 4a), whereas co-localization of TOPBP1 and MDC1 can be observed (figure 4b). Lastly, the arrow is also pointing out the co-localization of ecDNA and TOPBP1 (figure 5).

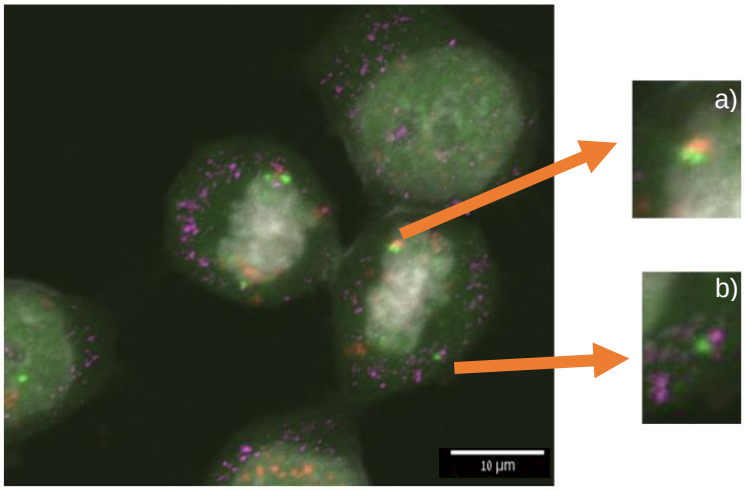

Figure 4: Control sample (no CX4945 present). The co-localization of ecDNA (red) and TOPBP1 (green) is shown in 4a. The co-localization of TOPBP1 (green) and MDC1 (purple) is shown in 4b

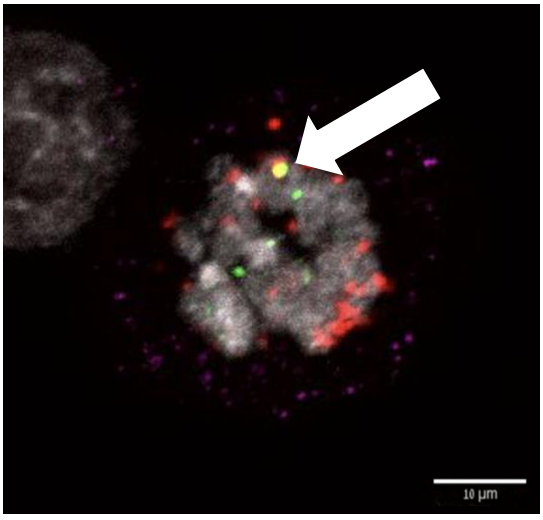

Figure 5: Control sample (no CX4945 present). The arrow is showing co-localization of ecDNA (red) and TOPBP1 (green)

Moving onwards, figure 6 sums up the tethering of ecDNA to chromosomes during mitosis with and without CX4945 treatment.

In the control sample, only '100% tethering' and 'high tethering' were detected. Here we observed greater percentage of 'high tethering' than the percentage of '100% tethering'. At 5µM and 10µM all three types of tethering were observed. 'Low tethering' increased as the concentrations of CX4945 increased, whereas 'high tethering' decreased as concentrations of CX4945 increased (figure 6).

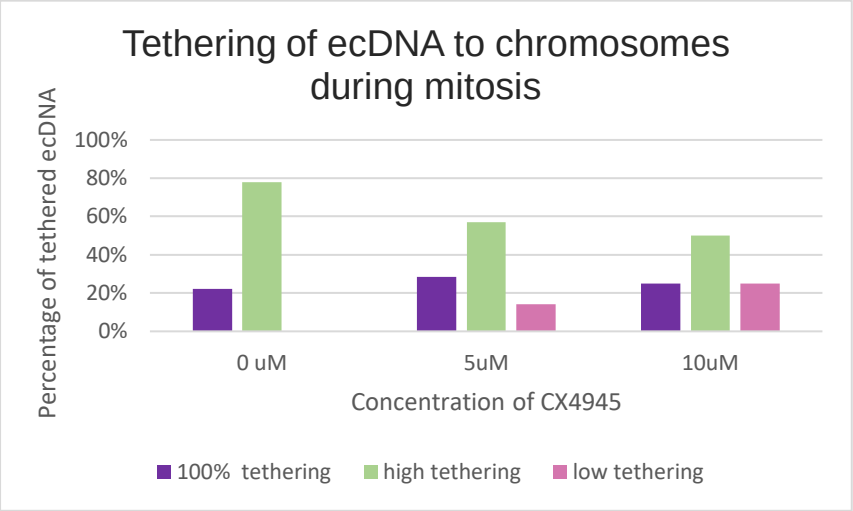

Figure 6: Tethering of ecDNA within three concentrations of CX4945

We then looked at a sample of cells treated with 10µM of CX4945, focusing on the co-localization of MDC1, TOPBP1 and ecDNA on the chromosomes (figure 7). We looked at images 7a) to 7c) and observed the individual staining of MDC1, TOPBP1 and ecDNA shown by the arrow. The final image 7d), shows the overlap of the first three photographs, to illustrate the co-localization of the three molecules. The results show an abnormality because the CX4945 is supposed to disrupt the interaction of MDC1 and TOPBP1, yet we see co-localization.

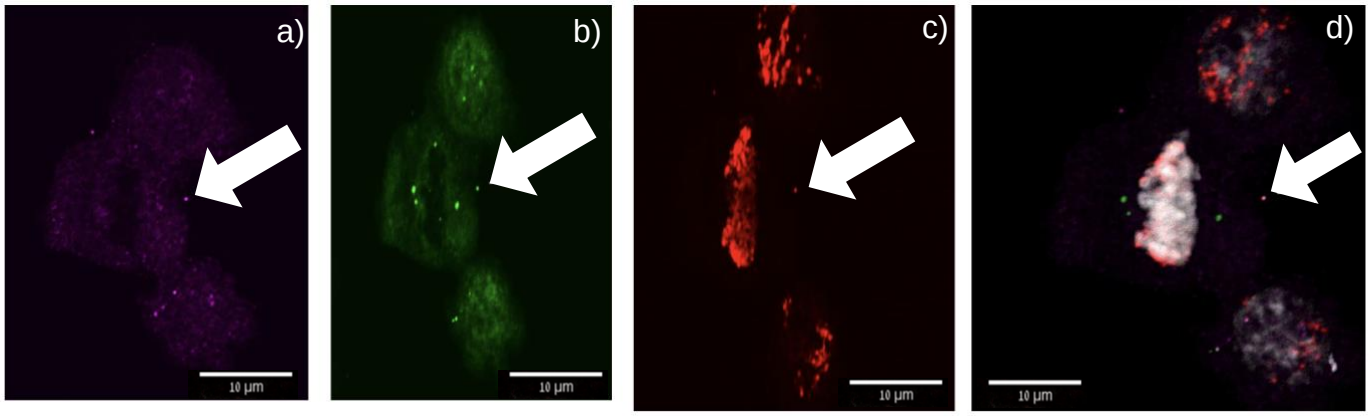

Figure 7: Cells treated with 10µM of CX4945. Co-localization of MDC1 (purple) (shown individually in 7a), TOPBP1 (green) (shown individually in 7b) and ecDNA (red) (shown individually in 7c) on chromosomes (white) emphasized by the arrow. Image 7d represents the final image with the first three pictures merged

We also looked at an example of broken chromosomes treated with 10µM of CX4945 (figure 8). We observe 'low tethering' of ecDNA in the broken chromosomes. We can see some of the ecDNA on the chromosomes and most of the ecDNA around them.

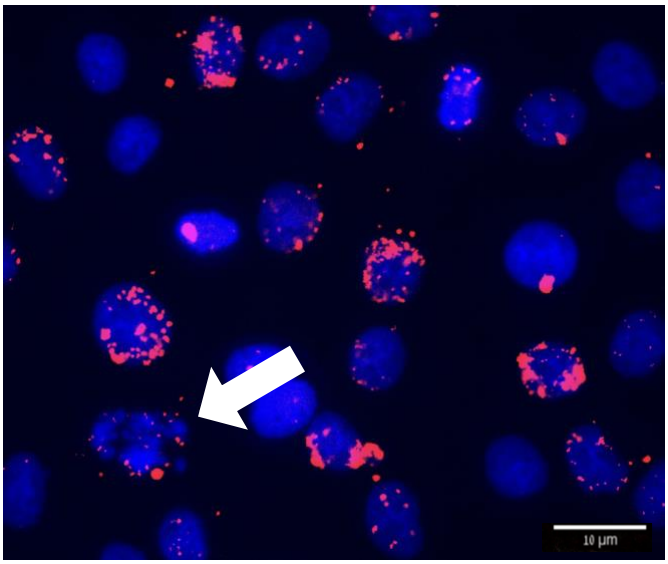

Figure 8: Cells treated with 10uM of CX4945. Tethering of ecDNA (red) on broken chromosomes (blue)

Lastly, we address the co-localization of MDC1, TOPBP1 and ecDNA overall in 0µM and 10µM of CX4945 in our samples (figure 9). The number of all observed co-localizations decreases as the concentration of CX4945 increases. As was expected we also observed decrease in TOPBP1 and MDC1 co-localization, but not completely.

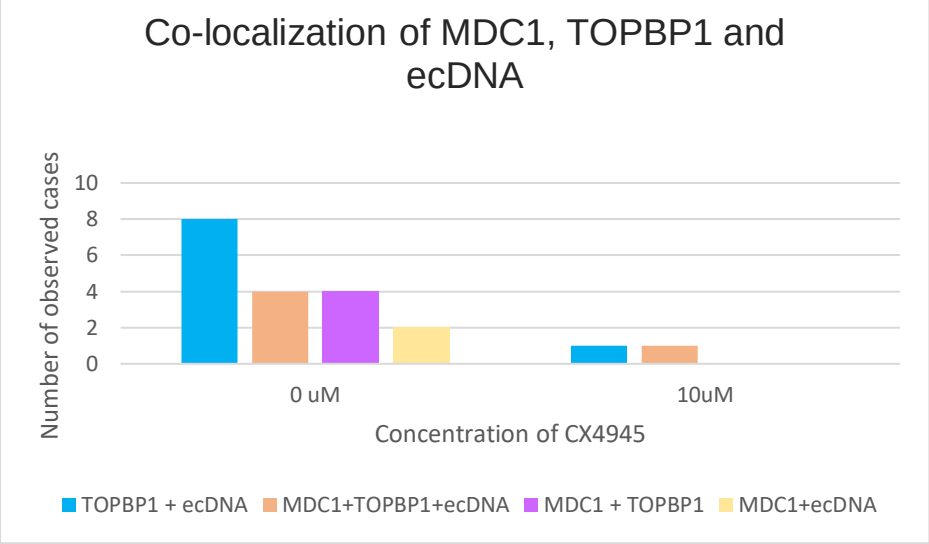

Figure 9: Co-localization of MDC1, TOPBP1 and ecDNA within 0µM and 10µM of CX4945

Discussion

We hypothesised that MDC1 and TOPBP1 proteins play a role in ecDNA tethering, as their complex is responsible for the DNA repair and maintaining chromosome stability during mitosis (6). Increased concentrations of CX4945 seem to interrupt interaction of TOPBP1 and MDC1 with each other, as well ecDNA and either one of the proteins, and lastly ecDNA with both proteins (figure 9). Consequently, according to the results we can observe less tethering

in higher concentrations of CX4945 (figure 6). However, in one case with the highest concentration of CX4945 we can still observe co-localization of TOPBP1-MDC1 complex and ecDNA which is unexpected (figure 7). In future studies increasing the concentration of the drug would be encouraged, as 10μM might not be fully sufficient to disrupt the interaction between TOPBP1-MDC1 complex and ecDNA.

In conclusion, these results suggest that the proteins TOPBP1 and MDC1 may be involved in tethering of ecDNA. Nevertheless, to confirm the hypothesis further investigation is required. In order to perform additional research in the similar direction it is essential to acknowledge the limitations of our study. Due to the time restriction, we worked on a relatively small sample size, which affected our ability to conduct statistical tests on the results. We also faced some difficulties while measuring the number of tethered ecDNA. The level of tethering was estimated, as it is hard to count the exact number of ecDNA. For future reference it might be better to use an AI tool. It is also worth mentioning that we had no 5μM sample at the co-localization graph (figure 9) as we lost it in due to technical issues in the process of preceding IF-FISH.

Even so, the research that we conducted is part of a larger project aiming to detect the mechanism of ecDNA inheritance. Our study delivered important evidence on the MDC1-TOPBP1 complex involvement in ecDNA tethering. Our results show that interruption of those proteins interaction resulted in decreased tethering. As ecDNA is one of the factors enabling tumours to maintain heterogeneity, it is crucial to understand the mechanism underlining its inheritance (12). We believe our research brought us closer to reaching that goal.

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