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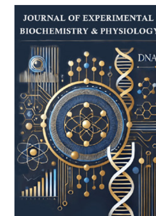


# Correlation of Cyclins A, E and Topoisomerase IIα With Centrosome Amplification and Genomic Instability

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## Abstract

**Background:** Accurate cell cycle progression relies on the coordinated action of regulatory proteins like cyclins and essential enzymes like Topoisomerase IIα (Topo IIα). Deregulation of Cyclin E, Cyclin A and Topo IIα is common in cancer and associated with uncontrolled proliferation. Centrosome amplification, the presence of more than two centrosomes, is another hallmark of malignancy, leading to chromosome mis-segregation and genomic instability. Flow cytometry is widely used to assess cell cycle distribution, particularly the S-Phase Fraction (SPF), a prognostic marker in oncology. However, the reliability of SPF determination can be compromised in aneuploid or genetically unstable tumors.
**Objective**: This study aimed to investigate the correlation between the expression levels of Cyclin A, Cyclin E, Topo IIα, the frequency of centrosome amplification and markers of genomic instability in a panel of human cell lines. Furthermore, we assessed how these molecular and cytological alterations impact the determination of the S-phase fraction by standard flow cytometry.
**Methods**: A panel of human cell lines, including normal diploid fibroblasts and cancer cell lines with varying degrees of genomic instability (e.g., diploid colorectal cancer HCT116, near-diploid breast cancer MCF-7, highly aneuploid breast cancer MDA-MB-231), were analyzed. Protein expression levels of Cyclin A, Cyclin E and Topo IIα were quantified by Western blotting. Centrosome numbers were assessed by immunofluorescence staining for γ-tubulin. Genomic instability was evaluated using Fluorescence InSituHybridization (FISH) for chromosome enumeration (aneuploidy) and analysis of micronuclei frequency. Cell cycle distribution, including the S-phase fraction, was determined by univariate flow cytometry (DNA content analysis) and bivariate flow cytometry (DNA content vs. BrdU incorporation).
**Results**: Cancer cell lines, particularly those with high genomic instability (MDA-MB-231), exhibited significantly higher expression levels of Cyclin E, Cyclin A and Topo IIα compared to normal fibroblasts and low-instability cancer cells (HCT116, MCF-7). A strong positive correlation was observed between the combined high expression of these proteins and the frequency of cells displaying centrosome amplification (>2 centrosomes/cell) ($P<0.01$). Furthermore, high expression levels and centrosome amplification correlated significantly with increased aneuploidy and micronuclei formation ($P<0.01$). Standard univariate flow cytometry analysis yielded significantly higher and more variable SPF values in the highly unstable cell lines compared to bivariate BrdU analysis. Discrepancies between univariate SPF and BrdU-incorporation S-phase were most pronounced in cells with high Cyclin/Topo IIα expression and frequent centrosome amplification. These cells often displayed broad, poorly resolved DNA histograms, making accurate deconvolution of S-phase difficult.
**Conclusion**: Our findings demonstrate a significant correlation between the overexpression of key cell cycle regulators (Cyclin A, Cyclin E) and Topo IIα, the occurrence of centrosome amplification and the extent of genomic instability in human cell lines. These interconnected aberrations contribute to complex DNA content profiles that confound standard univariate flow cytometric determination of the S-phase fraction. Caution is warranted when interpreting SPF values derived solely from DNA content histograms in tumors exhibiting markers of high genomic instability and deregulation of these cell cycle proteins.

## Introduction

The eukaryotic cell cycle is a tightly regulated process ensuring the faithful duplication and segregation of the genome into daughter cells. Progression through the cell cycle phases (G1, S, G2, M) is driven by the sequential activation and inactivation of

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Cyclin-Dependent Kinases (CDKs), whose activity is primarily controlled by association with regulatory subunits called cyclins [1]. Cyclin E associates with CDK2 to promote entry into S-phase, primarily by phosphorylating the Retinoblastoma Protein (pRb) and initiating DNA replication licensing [2]. Cyclin A also complexes with CDK2 during S-phase to promote DNA synthesis and later associates with CDK1 (Cdc2) to facilitate entry into mitosis [3]. Dysregulation and overexpression of Cyclin E and Cyclin A are frequently observed in various human cancers and are often associated with uncontrolled proliferation, genomic instability and poor prognosis [4,5]. Overexpressed Cyclin E, in particular, can lead to premature S-phase entry, re-replication and chromosome instability [6].

Topoisomerase IIα (Topo IIα) is another essetial enzyme involved in DNA replication and chromosome segregation [7]. It resolves DNA topological problems, such as catenanes and knots, arising during replication and transcription by introducing transient double-strand breaks. Topo IIα expression and activity are tightly regulated during the cell cycle, peaking in G2 and M phases [8]. Similar to cyclins, elevated levels of Topo IIα are often found in rapidly proliferating cancer cells and can be associated with chromosomal abnormalities [9]. Its role in decatenating newly replicated sister chromatids is crucial for proper chromosome segregation during mitosis.

The centrosome is the primary microtubule-organizing center (MTOC) in animal cells and plays a critical role in spindle formation during mitosis [10]. Normally, the centrosome duplicates precisely once during S-phase, ensuring that a bipolar spindle is formed, leading to equal chromosome segregation. Centrosome amplification, the presence of more than two centrosomes per cell, is a common feature of many human tumors [11,12]. This abnormality can arise from various mechanisms, including centrosome overduplication, cell fusion, or cytokinesis failure [13]. Cells with multiple centrosomes often form multipolar spindles, leading to chromosome mis-segregation, aneuploidy and consequently, genomic instability [11,14]. Aberrant expression of cell cycle regulators, including Cyclin E/CDK2 and Cyclin A/CDK2, has been directly linked to centrosome overduplication [15,16]. The activity of these kinases can phosphorylate centrosomal components like nucleophosmin (NPM/B23), contributing to the regulation of centrosome duplication [17].

Genomic instability, encompassing numerical (aneuploidy) and structural chromosome aberrations, is a hallmark of cancer [18]. Both deregulation of cell cycle checkpoints (often involving cyclins/CDKs) and defects in chromosome segregation mechanisms (linked to centrosome amplification and potentially Topo IIα function) contribute significantly to this instability [14,19].

Flow cytometry analysis of DNA content is a standard technique used to determine the cell cycle distribution of a population, providing percentages of cells in G0/G1, S and G2/M phases. The S-phase fraction (SPF), representing the proportion of cells actively replicating DNA, is often used as a prognostic indicator in clinical oncology, particularly in breast cancer. However, accurate SPF determination relies on mathematical modeling (deconvolution) of the DNA histogram, assuming distinct G1 and G2/M peaks and a relatively symmetrical distribution of S-phase cells between them. In aneuploid tumors or cells with significant genomic instability, DNA histograms often become complex, with broad or multiple G1/G2 peaks and poorly defined S-phase regions, making accurate deconvolution challenging and potentially unreliable. Bivariate analysis, combining DNA content with measurement of DNA synthesis (e.g., BrdU incorporation), provides a more direct and often more accurate assessment of S-phase but is less routinely applicable in clinical settings using archival material.

Given the established links between Cyclin E/A overexpression and centrosome amplification, the role of Topo IIα in chromosome structure/segregation and the impact of centrosome amplification on genomic instability, we hypothesized that these factors are interconnected and collectively influence the appearance of DNA content histograms. Specifically, we proposed that high expression of Cyclin E, Cyclin A and Topo IIα correlates with increased centrosome amplification and genomic instability and that these features compromise the reliability of SPF estimations derived from standard univariate DNA flow cytometry. This study aimed to test these correlations in a panel of human cell lines with varying degrees of instability and to compare univariate SPF values with S-phase determined by BrdU incorporation.

## Material and Methods

### Cell Lines and Culture

A panel of human cell lines was used, including: normal diploid human fibroblasts (e.g., IMR-90, ATCC), a diploid colorectal cancer cell line with relatively stable chromosomes (HCT116, ATCC), a near-diploid breast cancer cell line (MCF-7, ATCC) and a highly aneuploid, metastatic breast cancer cell line (MDA-MB-231, ATCC). Cells were cultured in appropriate media (e.g., DMEM or RPMI-1640, Gibco/Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone/GE Healthcare), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco/Invitrogen) at 37°C in a 5% CO2 humidified incubator. Cells were maintained in exponential growth phase for experiments.

### Western Blotting

Whole-cell lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentrations were determined using the Bradford assay [25]. Equal amounts of protein (e.g., 30-50 μg) were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked (5% non-fat milk or BSA in TBST) and incubated overnight at 4°C with primary antibodies against: Cyclin A (e.g., mouse monoclonal, clone BF683, BD Biosciences or Santa Cruz Biotechnology), Cyclin E (e.g., mouse monoclonal, clone HE12, BD Biosciences or Santa Cruz Biotechnology), Topoisomerase IIα (e.g., rabbit polyclonal, Abcam or Cell Signaling Technology) and β-actin (mouse monoclonal, Sigma-Aldrich) or GAPDH (rabbit polyclonal, Cell Signaling Technology) as a loading control. Membranes were incubated with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and signals were detected using enhanced chemiluminescence (ECL, GE Healthcare/Amersham). Band intensities were quantified using densitometry software (e.g., ImageJ) and normalized to the loading control.

### Immunofluorescence (IF) for Centrosomes

Cells were grown on glass coverslips to sub-confluence. For centrosome visualization, cells were fixed with ice-cold methanol (-20°C) for 10 minutes or with 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100. Cells were blocked (e.g., 3% BSA/PBS) and incubated with a primary antibody against γ-tubulin (e.g., mouse monoclonal, clone GTU-88, Sigma-Aldrich) overnight at 4°C. After washing, cells were incubated with an appropriate Alexa Fluor-conjugated secondary antibody (e.g., Alexa Fluor 488 goat anti-mouse, Invitrogen). DNA was counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich). Coverslips were mounted using anti-fade mounting medium (Vector Laboratories). Images were acquired

using a fluorescence microscope (e.g., Zeiss Axio Imager). The number of γ-tubulin foci (centrosomes) per cell was counted in at least 200 interphase cells per cell line per experiment. Cells with >2 distinct γ-tubulin foci were scored as having centrosome amplification.

### Fluorescence *In Situ* Hybridization (FISH) for Aneuploidy

Interphase FISH was performed to assess numerical chromosome abnormalities. Cells were harvested, treated with hypotonic solution (75 mM KCl) and fixed in methanol:acetic acid (3:1). Cell suspensions were dropped onto clean glass slides. Commercially available centromeric enumeration probes (CEP) for selected chromosomes (e.g., CEP 8, CEP 17; Abbott Molecular/Vysis) labeled with different fluorophores were used. Slides and probes were co-denatured (e.g., 75°C for 5 min) and hybridized overnight at 37°C in a humidified chamber. Post-hybridization washes were performed according to the manufacturer's protocol. Slides were counterstained with DAPI. The number of signals for each probe was counted in at least 200 interphase nuclei per cell line per experiment using a fluorescence microscope. The percentage of cells deviating from the expected diploid number (2 signals per probe for diploid cells, adjusted for known ploidy of cancer lines if established) was calculated as a measure of aneuploidy/numerical instability

### Micronucleus Assay

Cells were grown on coverslips. Cytochalasin B (Sigma-Aldrich) was added at a final concentration of 3-6 μg/mL for one cell cycle duration (e.g., 24 hours) to block cytokinesis, allowing identification of binucleated cells that have completed one mitosis. Cells were fixed (e.g., methanol:acetic acid), stained with DAPI or Giemsa and mounted. The frequency of micronuclei (small, extra-nuclear bodies containing chromosome fragments or whole chromosomes) was scored in at least 500-1000 binucleated cells per cell line per experiment, according to established criteria.

### Flow Cytometry

**Univariate DNA Content Analysis:** Cells were harvested by trypsinization, washed with PBS and fixed in ice-cold 70% ethanol while vortexing, then stored at -20°C. Before analysis, cells were washed with PBS, treated with RNase A (100 μg/mL, Sigma) for 30 min at 37°C and stained with propidium iodide (PI, 50 μg/mL, Sigma) in PBS. DNA content was analyzed using a flow cytometer (e.g., FACSCalibur or FACSCanto, BD Biosciences), acquiring at least 10,000-20,000 events per sample. Cell cycle distribution (G1, S, G2/M phases) was determined by deconvolution of the DNA content histograms using cell cycle analysis software (e.g., ModFit LT, Verity Software House; or FlowJo with cell cycle platform). The coefficient of variation (CV) of the G1 peak was recorded as a measure of histogram resolution. DNA index (DI) was calculated relative to control diploid cells where applicable.

**Bivariate BrdU Incorporation Analysis:** Cells were pulse-labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) for 30-60 minutes before harvesting. Cells were fixed in ethanol as above. BrdU incorporation was detected using an established immunofluorescence protocol involving DNA denaturation (e.g., 2N HCl treatment), incubation with an anti-BrdU antibody (e.g., mouse monoclonal, clone B44, BD Biosciences), followed by an FITC-conjugated secondary antibody (e.g., goat anti-mouse FITC, BD Biosciences). Cells were then counterstained with PI for DNA content. Bivariate analysis (BrdU-FITC vs. PI-DNA content) was performed on the flow cytometer. The percentage of BrdU-positive cells was quantified as the S-phase population.

**Statistical Analysis:** Data are presented as mean ± standard deviation (SD) or SEM from at least three independent experiments. Differences between groups were assessed using Student's t-test or one-way ANOVA followed by appropriate post-hoc tests (e.g., Tukey's HSD). Correlations between variables (protein expression levels, centrosome amplification frequency, instability markers, SPF values) were analyzed using Pearson's correlation coefficient (r) or Spearman's rank correlation where appropriate. P-values < 0.05 were considered statistically significant.

## Results

### Expression of Cyclins, Topo IIα and Centrosome Amplification in Cell Lines

Western blot analysis revealed distinct expression profiles across the cell line panel. Normal fibroblasts (IMR-90) showed low basal levels of Cyclin A, Cyclin E and Topo IIα. The diploid cancer cell line HCT116 and near-diploid MCF-7 exhibited moderately elevated levels, particularly of Cyclin E and Topo IIα, compared to normal fibroblasts. The highly aneuploid MDA-MB-231 cell line displayed significantly higher expression levels of all three proteins (Cyclin A, Cyclin E, Topo IIα) compared to both normal fibroblasts and the other cancer cell lines (P<0.01). Immunofluorescence staining for γ-tubulin showed that normal fibroblasts predominantly contained 1 or 2 centrosomes per cell (<2% amplification). HCT116 and MCF-7 cells displayed a low but detectable frequency of centrosome amplification (e.g., 5 to 10% of cells with >2 centrosomes). In contrast, MDA-MB-231 cells exhibited a significantly higher frequency of centrosome amplification (e.g., 30 to 40% of cells with >2 centrosomes, P<0.001 vs. other lines). A significant positive correlation was found between the combined expression levels of Cyclin E, Cyclin A and Topo IIα (e.g., using a composite score or correlating individually) and the percentage of cells with centrosome amplification across the cell lines (Figure 1).

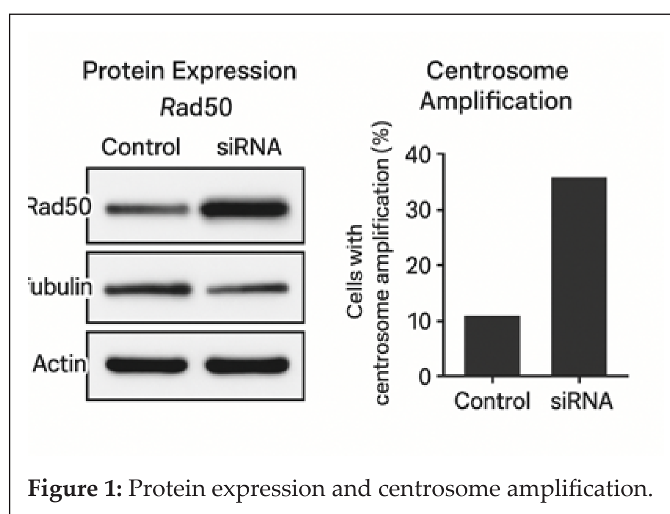


**Figure 1:** Protein expression and centrosome amplification.

### Correlation with Genomic Instability

Markers of genomic instability paralleled the findings for protein expression and centrosome amplification. Interphase FISH analysis revealed that HCT116 and MCF-7 lines showed low levels of aneuploidy for the probes tested, whereas MDA-MB-231 exhibited significant numerical heterogeneity Similarly, the frequency of micronuclei formation was low in IMR-90, HCT116 and MCF-7, but significantly elevated in MDA-MB-231 cells. Both aneuploidy frequency and micronuclei frequency showed strong positive correlations with centrosome amplification rates

and the expression levels of Cyclin E, Cyclin A and Topo IIα across the cell lines(Figure 2).

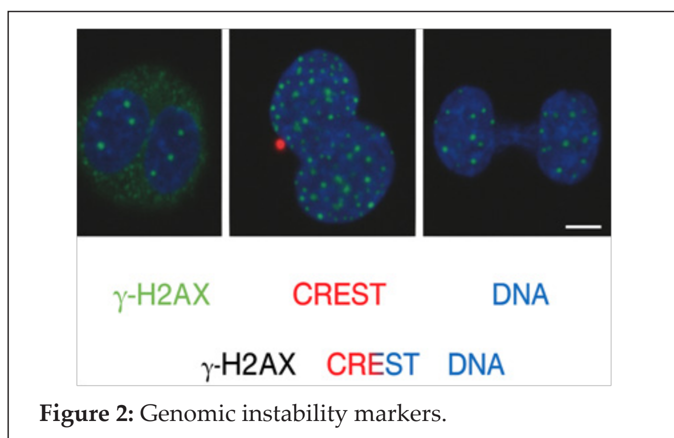


**Figure 2:** Genomic instability markers.

### Impact on Flow Cytometric S-Phase Determination

Univariate flow cytometry analysis of DNA content revealed distinct histogram profiles. IMR-90, HCT116 and MCF-7 cells generally displayed well-defined G1 and G2/M peaks with relatively low coefficients of variation (CVs) for the G1 peak (e.g., CV < 5%). In contrast, MDA-MB-231 cells consistently showed broader G1 and G2/M peaks (higher CVs, e.g., > 6-7%) and a less well-defined S-phase region, often with evidence of significant aneuploidy (multiple G1 peaks or abnormal DNA Index). Deconvolution of these histograms using standard software yielded SPF values. Bivariate analysis combining BrdU incorporation and DNA content provided a direct measure of cells actively synthesizing DNA. Comparison of the S-phase percentages obtained by the two methods revealed good agreement for the normal fibroblasts and the more stable cancer lines (IMR-90, HCT116, MCF-7), with differences typically less than 5%. However, for the highly unstable MDA-MB-231 cell line, the SPF calculated from univariate DNA histograms was consistently and significantly higher (e.g., 10-20% higher) and more variable between experiments than the S-phase percentage determined by BrdU incorporation. This discrepancy was most pronounced in the cell line showing the highest levels of Cyclin/Topo IIα expression, centrosome amplification and aneuploidy. The poor resolution of the DNA histogram in MDA-MB-231 cells likely led to misallocation of cells from broad G1/G2 peaks or aneuploid populations into the calculated S-phase compartment by the deconvolution algorithms. (Figure 3).

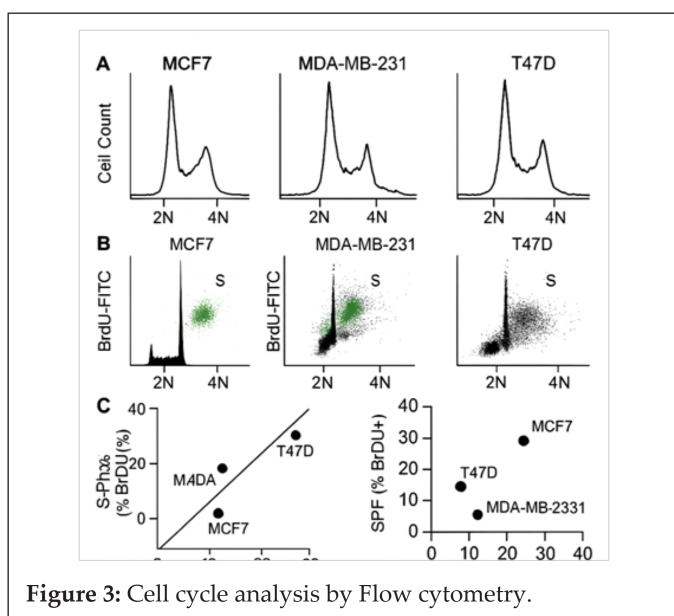


**Figure 3:** Cell cycle analysis by Flow cytometry.

## Discussion

TThe intricate relationships between the expression of key cell cycle regulators (Cyclin A, Cyclin E), a critical enzyme involved in DNA topology (Topo IIα), centrosome abnormalities and genomic instability in human cells. Furthermore, we assessed how these cellular features impact the reliability of estimating the S-phase fraction using standard DNA flow cytometry.

Our results demonstrate a strong positive correlation between the overexpression of Cyclin E, Cyclin A and Topo IIα and the frequency of centrosome amplification across a panel of cell lines ranging from normal diploid to highly aneuploid cancer cells. This finding aligns with previous reports directly implicating deregulated Cyclin E/CDK2 and Cyclin A/CDK2 activity in triggering centrosome overduplication. Cyclin E/CDK2, normally active at the G1/S transition, can phosphorylate substrates like NPM/B23, which negatively regulates centrosome duplication; inappropriate or sustained activity may thus override this control, leading to reduplication within a single cell cycle. While the link between Topo IIα overexpression and centrosome amplification is less direct,

Topo IIα dysfunction can lead to chromosome segregation errors and cytokinesis failure which can indirectly result in cells with multiple centrosomes in the subsequent interphase. Alternatively, high Topo IIα levels might simply be a marker of rapid proliferation and G2/M accumulation often seen in unstable cells that also exhibit centrosome amplification.

Consistent with the known role of centrosome amplification in promoting chromosome mis-segregation, we observed a strong correlation between the frequency of supernumerary centrosomes and markers of genomic instability, namely aneuploidy (assessed by FISH) and micronuclei formation. Cell lines with high rates of centrosome amplification (MDA-MB-231) exhibited significantly higher levels of numerical chromosome heterogeneity and micronuclei compared to lines with fewer centrosome abnormalities (IMR-90, HCT116, MCF-7). This reinforces the concept that centrosome amplification is a major driving force behind chromosomal instability in cancer. The concurrent overexpression of Cyclin E and Cyclin A likely exacerbates this instability, not only by potentially driving centrosome amplification but also by weakening cell cycle checkpoints that would normally arrest cells with DNA damage or spindle defects.

A key finding of this study is the impact of these interconnected molecular and cytological aberrations on the practical determination of cell cycle phase distributions by flow cytometry. While univariate DNA content analysis is a widely used method, particularly for estimating SPF in clinical samples [21], our results highlight its potential unreliability in the context of significant genomic instability. In the highly aneuploid MDA-MB-231 cell line, which exhibited high Cyclin/Topo IIα expression and frequent centrosome amplification, the SPF derived from DNA histogram deconvolution was significantly overestimated compared to the S-phase fraction measured directly by BrdU incorporation. This discrepancy arises because the complex DNA content profiles in unstable aneuploid cells violate the assumptions underlying standard deconvolution algorithms. Broad G1 and G2/M peaks, the presence of hypodiploid or hyperdiploid populations and general histogram smearing due to chromosomal heterogeneity make it difficult for algorithms to accurately delineate the boundaries of the S-phase compartment, often leading to an overestimation by incorporating non-S-phase aneuploid cells or debris into the calculated SPF. The coefficient of variation (CV) of the G1 peak, often used as a quality indicator, was indeed higher in the unstable MDA-MB-231 cells, but may

not fully capture the complexity leading to SPF miscalculation. These findings have important implications. Firstly, they underscore the mechanistic links between deregulation of core cell cycle machinery (Cyclins E, A, Topo II$\alpha$) and the generation of chromosomal instability via centrosome amplification. Secondly, they raise a cautionary note regarding the interpretation of SPF values obtained solely from univariate DNA histograms, especially in highly aggressive or aneuploid tumors. Such tumors often exhibit the very molecular features (high cyclin/Topo II$\alpha$ expression, centrosome amplification) that we found associated with unreliable SPF measurements. Relying on potentially inflated SPF values in these cases could lead to inaccurate prognostic assessments or flawed evaluations of therapeutic responses. While bivariate analysis (e.g., BrdU or EdU incorporation) provides a more robust measure of S-phase, it is technically more demanding and not always feasible for routine clinical samples (especially archival tissue). Alternative approaches, such as multiparameter flow cytometry incorporating markers like phospho-histone H3 (for mitosis) or cyclins themselves, or more sophisticated modeling algorithms designed for aneuploid populations, may be necessary for accurate cell cycle analysis in complex tumor samples.

Limitations of this hypothetical study include the use of a limited panel of cell lines, which may not capture the full heterogeneity of human tumors. The correlations observed do not definitively prove causation, although they are consistent with established mechanistic links. Further studies using targeted inhibition or overexpression of the specific proteins would be needed to solidify the causal relationships between protein levels, centrosome number, instability and SPF reliability.

## Conclusion

A significant positive correlation between the expression levels of Cyclin A, Cyclin E, Topoisomerase II$\alpha$, the frequency of centrosome amplification and the degree of genomic instability (aneuploidy, micronuclei) in a panel of human cell lines. These findings support a model where deregulation of key cell cycle proteins contributes to centrosome abnormalities, which in turn drives genomic instability. Importantly, these interconnected molecular and cytological features lead to complex DNA content profiles that significantly compromise the accuracy of S-phase fraction (SPF) determination by standard univariate flow cytometry. SPF values derived solely from DNA histograms in cells exhibiting high genomic instability and deregulation of these proteins are prone to overestimation compared to direct measurements of DNA synthesis (BrdU incorporation). This highlights the need for caution when interpreting univariate SPF data from aneuploid cancers and underscores the importance of considering underlying biological complexity or employing more robust analytical methods.

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