Expression Of Cell Cycle Markers in HEK293 and HT-29 Cell Lines Associated with PLK*1* and *INCENP* Expression

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ABSTRACT

Every cancer cell has fundamental properties that confers them the ability of growing uncontrollably and adapt to their environment. Genomic instability (GI) is one of these characteristics and chromosomal instability is one of its main forms. Instability is reached because there are altered mechanisms that causes chromosome missegregation resulting in changes of chromosomes number and structure. The main mechanisms by which chromosomes are affected are by dysregulation of cell cycle checkpoint pathways. Certain genes are altered by this instability, impacting the cell cycle progression. Various circuits are proposed in Suescum and collaborators instability model, that represents the interactions between these proteins. In this study, the expression of two regulatory genes [*INCENP* and *PLK1*], present in the model, and linked with chromosomal instability is measured by qPCR analysis in HEK293 [embryonic kidney line] and HT-29 [colon adenocarcinoma model]. Additionally, the expression of key cell cycle genes [*CDK1, CDC20, AURKB*] is measured in the same cell lines to compare their expression regarding the dysregulation of *PLK1* and *INCENP* genes. The results showed a decrease in the expression of studied genes in HT29 cell line in comparison to HEK293. Suggesting that evaluated cancer cells have reduced levels of expression in important mitotic genes.

Keywords: *Cancer, cell cycle, genomic instability, chromosome missegregation, gene expression, HEK293, HT29.*

INTRODUCTION

Cancer is a complex disease that involves uncontrolled growth and spread of abnormal cells (neoplastic cells) in the body. Most abnormal cells contain different mutations in multiple functional pathways inside the cell providing each of them fundamental characteristic that are common in most cancer types. These functional capabilities are acquired to help each cancer cell to survive, proliferate, and disseminate. Therefore, cancer involves dynamic changes within the cell transforming them to neoplastic cells (Hanahan and Weinberg, 2000). Throughout the years, cancer research has progressed, and new hallmarks have been identified, modifying their original formulation (Hanahan, 2011, 2022). One of the most significant hallmarks is sustained proliferative signaling. Cancer cells have a persistent drive to divide and replicate, allowing them to grow and form tumors. Additionally, cancer cells can evade growth suppressors and resist cell death, allowing them to avoid programmed cell death and to

ignore signals that would normally stop cell division (Hanahan 2011). Another important trait is enabling replicative immortality, meaning that cancer cells can continue dividing indefinitely. This contributes to the growth and spread of tumors. Cancer cells also induce angiogenesis, which is the growth of new blood vessels to supply nutrients and oxygen to tumors (Hanahan and Weinberg, 2000). Furthermore, they can activate invasion and metastasis, breaking away from the primary tumor and spreading to other parts of the body. Cancer cells can also deregulate cellular energetics or cellular metabolism, altering their metabolism to obtain energy and nutrients in abnormal ways, which allows them to survive and grow under conditions that would normally be toxic. Another trait of cancer cells is their ability to avoid immune destruction, evading the immune system, which would normally recognize and eliminate abnormal cells. (Hanahan and Weinberg, 2000; Hanahan, 2011, 2022).

Apart from the aforementioned hallmarks, tumor-promoting inflammation is an enabling characteristic along with genome instability and mutation. Together, they are fundamentally involved in activating the functional capabilities necessary for tumor growth and progression (Hanahan, 2022). Every cell in an organism must cope with mutation events, but cancer cells tend to have genetic changes, and mutations at a rate higher than normal. These mutations are generally in multiple growth and proliferation promoting pathways (Pfau and Amon, 2012), affecting the function of important regulation mechanisms like damage response, DNA repair, cell cycle checkpoints and mitotic processes (Yao and Dai, 2014). Genomic instability can occur at different levels, including at the level of individual genes, chromosomes, or the entire genome. There are several mechanisms that can cause genomic instability, including errors in DNA replication, exposure to certain environmental agents such as radiation or chemicals, and defects in DNA repair mechanisms. When these mechanisms are disrupted or impaired, genetic changes can accumulate over time, leading to genomic instability. There are various forms for genomic instability, most cancers exhibit chromosomal instability (CIN) which represents the structural changes at a high rate of chromosome number and structure in cancer cells (Negrini *et al.,* 2010). This changes in chromosomes are due to missegregation and fragmentation during mitosis, resulting in major changes like chromosomic deletions and translocations, affecting ploidy levels (Ippolito *et al*., 2021). Additionally, extra chromosomes may cause overexpression of some genes that induce tumorigenesis (Chandhok and Pellman, 2009).
Three types of genomic instability have been identified in colon cancer: Microsatellite instability (MSI), chromosome instability (CIN), and chromosomal translocations. Microsatellite instability results from an inactivation of mismatch repair-associated mechanisms. CIN results from deregulation of DNA replication checkpoints and mitotic spindle checkpoints. Therefore, all colon cancers display either MSI or CIN suggesting that genomic instability is central in colon cancer formation. MSI and CIN develop different kind of aberrations in cells, MSI cancer cells are diploid and have normal rates of chromosomal changes. On the other hand, CIN cells are aneuploid and have increased rates of chromosomal gains

and losses (Grady M, 2004). Therefore, the loss of genomic stability appears to be a key molecular and pathogenic step that occurs early in the tumorigenesis process of colonic adenocarcinoma cells.
To acquire the mutant genes that promote tumorigenesis cancer cells often increase their mutation rate (Negrini *et al*., 2010; Salk *et al*., 2010). Generally, the genes altered to begin this process are very specific and are essential to promote metabolic abnormalities in the cells. These specific genes are responsible of controlling the function of certain checkpoints in the cell cycle. According to various studies, specific genes like *P53, PLK1* and *KIF2* are involved in the progression of genomic instability in cancer cells. The proteins synthesized by these genes are present in multiple biological processes that are key in maintaining stability within the genome. The presence of each is essential during specific mitotic events, and many of them depend on each other to activate. In the mathematical model constructed by Suescum and collaborators, it is possible to analyze PLK1 protein interactions with mitotic biological processes. Likewise, it models key proteins involved in cell cycle regulation, mitotic progression, and genes important for *PLK1*-mediated genomic instability events. Polo-like kinase 1 [*PLK1]* has typically been considered a proto-oncogene relevant for cell cycle progression, where it is commonly found to be overexpressed and shows strong correlation with carcinogenesis events and checkpoint dysregulation. Other important genes mentioned in the model are *KI2F* and *INCENP* genes, both are important for microtubule-chromosomal dynamics, microtubule interactions, spindle checkpoint, mitotic exit, and proper chromosome segregation (Suescum *et al.,* 2023; Shannon and Salmon, 2002).

In this study we aim to evaluate whether *INCENP* and *PLK1* differential gene expression is associated with transcriptional changes in cell cycle markers such as *CDK1, AURKB and CDC20.*

METHODOLOGY

Cell culture and RNA extraction

Cultures of HEK293 [embryonic kidney line] and HT-29 [colon adenocarcinoma model] cell lines were used in the present study. Both cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. For both cell lines, approximately 100,000 cells were seeded in 4 ml of medium in a 25 cm^2 culture flask and incubated at 37 °C with 5% CO_2 until the cultures reached 80% confluence. At that time, the necessary passages were performed to ensure the survival and viability of the culture for the duration of the experiment.

Real time qPCR experiments

RNA was extracted by triplicates (biological replicates) from different HEK293 and HT29 cultures. The RNeasy Mini Kit [Qiagen®] was used for RNA extraction, and the company's procedural instructions were followed. The quantity and quality of the extracted RNA were determined using a NanoPhotometer® N120 (IMPLEN-Germany). Subsequently, 1 µg of RNA obtained was converted to

complementary DNA [cDNA] using the Transcriptor Universal cDNA Master Mix kit [Merck S.A., Darmstadt, Germany] according to the manufacturer's instructions. As a template, 2 ng of each cDNA sample were amplified in 10 µl of reaction mix buffer containing 5 µl of SYBR® Green qPCR Ready mix™ (Merck S.A., Germany), and 1 µl of forward and reverse primers in a final concentration of 2.5 µM. The reaction mixes contained the intercalating dye SYBR® Green I for cDNA detection and analysis. The thermal cycling protocol involved a pre-incubation step at 95°C for 10 min, followed by three amplification steps of 45 cycles (95°C for 30 s, 65°C for 30s and 72°C for 35 s). The program ended with a Tm calling analysis necessary for calculating melting curves and peaks. We used *YWHAZ* gene as normalization factor for all the experiments. Relative expression was calculated using the 2-ΔΔCt relative quantification method (Livak & Schmittgen 2001), contrasting the HEK293 cell line (control) with the HT29 line. The expression of the evaluated genes was normalized using the *YWHAZ* gene as a normalizer. Finally, paired T-student tests were performed to determine significant differences ($p \leq 0.05$) between gene expression values. The primer sequences followed in this study are depicted in table 1.

Table 1. Represents each gene sequence used in both cell lines HEK293 and HT29.

Cell lines	Gene	Primer
HEK293, HT29	PLK1	FW 5´-GACCTCAAGCTGGGCAACCTCT-3´
		RV 5´-GACCACACATCCACCTCAAAACTG-3´
	INCENP	FW 5´- GAGGAGGCTGAGCGCATGTT-3´
		RV 5´-GACTTTCTGCGGGATAACCTTTTC-3´
	CDK1	FW 5´-GGATGTGCTTATGCAGGATTCC-3´
		RV 5´- CATGTACTGACCAGGAGGGATAG-3´
	CDC20	FW 5´-GACCACTCCTAGCAAACCTGG-3´
		RV 5´-GGGCGTCTGGCTGTTTTCA-3´
	AURKB	FW 5´-CGCAGAGAGATCGAAATCCAGG-3´
		RV 5´-CCCTTGAGCCCTAAGAGCAGATTT-3´
	YWHAZ	FW 5´-ACTTTTGGTACATTGTGGCTTCAA-3´
		RV 5´ -CCGCCAGGACAAACCAGTAT-3´

RESULTS

Expression analysis of key genes

Real-time PCR provided a clear comparison between the target genes in the two different cell lines. Significant differences were found in the expression levels between cell lines. Every gene had a lower relative expression in HT29 cell lines than HEK293. According to the results, *PLK1* expression was depleted 36% in HT29 cells in comparison to HEK293, nevertheless this difference was not statistically significant. However, there was a significant statistical difference between the values of every other gene in each cell line. *INCENP* expression was depleted 56% in HT29 cells and showed a significant difference of 0,041. On the other hand, *CDK1* showed a much higher significant difference now that its significant value is 0,046 less than 0,05. Its expression results show it was depleted 73% in HT29 cells in contrast to HEK293 cells. *CDC20* expression was 72% higher in HEK293 cells than HT29, showing a

significant difference in expression values. Lastly, *AURKB* showed the highest significant differences between each cell line value. The value determined by T-student test was 0,049 less than 0,05. *AURKB* expression was depleted 87% in HT29 cells in comparison to HEK293.

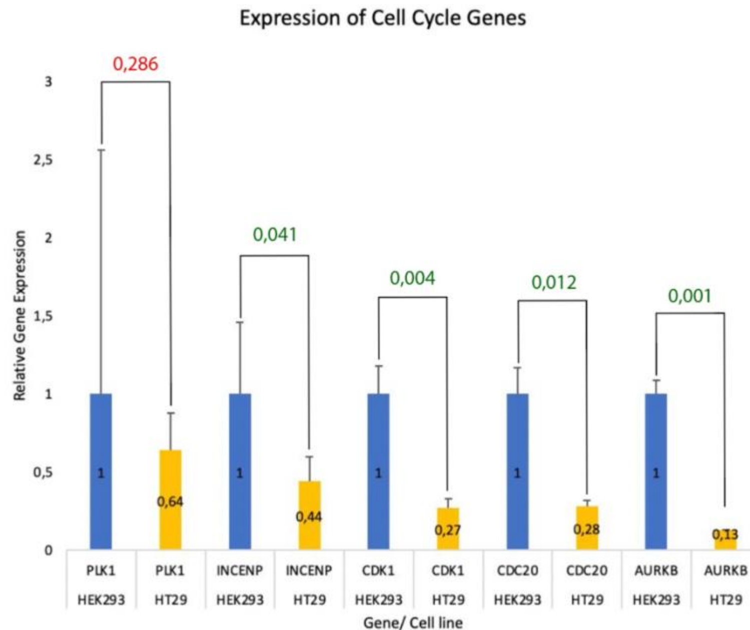

Fig 2. Relative expression levels of the five evaluated genes in HEK293 and HT29 cell lines. HEK293 samples were used as a calibrator and set to 1.0 for calculating the relative expression. HT29 cell lines showed a lower expression in all measured genes in comparison to HEK293 genes expression. Green and red values correspond to each result of the paired T-student test to determine significant differences ($p \leq 0.05$).

DISCUSSION

Genomic instability (GI) is a characteristic in all human cancers, and chromosomal instability is one of its main forms. It correlates with high rates in changes of chromosome structure and number. These changes are due to mechanisms that involve chromosome missegregation during cell cycle. In this study the expression of cell-cycle-related genes was measured to evaluate the impact of genomic instability genes dysregulation. In the cell cycle, there are various interactions between all the proteins that are encoded by the target genes used in this study. Although the results showed a decrease in *PLK1* and *INCENP* genes expression, cell dynamics are affected, and cell cycle circuits related to microtubule-kinetochore assembly may vary. The two studied cell lines were crucial for the comparison now that HEK293 serves as a control cell line, with standard expression levels of this genes. On the contrary, HT29 is a colorectal adenocarcinoma cell line which should present abnormal expression levels.

Broadly, the genomic instability model proposed by Suescum *et al.,* identifies three interacting circuits that can potentially induce a genomic instability event as a product of *PLK1* deregulation. The first circuit is the chromosomal passenger complex (CPC) that works along with INCENP, BIRC5, AURKB and

CDCA8 proteins. The second circuit involves microtubule-binding to chromosomes and mitotic checkpoint regulation. Lastly, the third circuit corresponds to anaphase transition and ESPL1 protein activity. As a result, the model shows different interactions between proteins that may play a role in genomic instability. One of these proteins is INCENP*,* which acts as a binding structure inside each complex. First and foremost, INCENP is fundamental in CPC now that it is the main structural base where other proteins of the complex bind into (AURBK, BIRC5 and CDCA8). This complex is located in the centrosomes of mitotic spindles, its activity is stimulated by PLK1 protein, and its main role is to help in the attachment and dissociation of microtubules and kinetochores (Carmena *et al.,* 2012). When PLK1 activates the complex, AURBK is strongly activated upon binding with INCENP whether BIRC5 nor CDCA8 are present (Honda *et al.*,2003). This activation takes place when AURBK phosphorylates an evolutionary conserved motif (TSS) within the C terminal region of INCENP called IN-box (Bishop and Schumacher, 2002), suggesting that INCENP is an activator and a substrate of AURBK. This phosphorylation is required for full activation of the complex, and without its proper functionality centromeres wouldn't be able to localize during metaphase and relocalize in the midzone of the spindle during anaphase (Honda *et al.,* 2003).
On the other hand, INCENP works along with AURBK not only for CPC functionality, now that both proteins form a complex of their own that phosphorylates PLK1 and regulates COHESIN. INCENP/AURBK phosphorylate PLK1 to activate its biologic functions such as centrosome segregation. Likewise, INCENP/AURBK regulates COHESIN, a protein that attaches chromosomes before they segregate and prevents them from a premature segregation (Suescum *et al.,* 2023).
Regarding the second interacting circuit presented by the model, there is a mitotic checkpoint system that ensures the proper chromosome segregation in mitosis. When this checkpoint is activated, there is an accumulation of the MCC complex. This complex is formed by the binding of two inner complexes: CDC20 protein with C-MAD2 protein and BUB1 protein with BUB3. When MCC is activated, it inhibits the ubiquitin ligase Anaphase promoting complex APC/C. Consequently, when MCC is turned off, APC/C is activated, and it initiates anaphase by cycling B degradation (Kaisari *et al*., 2019). For MCC activation, BUB1-BUB3 complex is phosphorylated by CDK1, and it binds to PLK1. Later, BUB1-BUB3 complex recruits the other protein complexes in MCC (BUBR1-BUB3, MAD2-CDC20) to the kinetochores. Through the interactions of the inner complexes, MCC is activated inhibiting APC/C activity. Alongside, BUB1 binds with PLK1 to phosphorylate CDC20 and induce APC/C inhibition. Both mechanisms are required for proper spindle checkpoint signaling. When either of this mechanism is deactivated, checkpoint defects may result in chromosome missegregation and aneuploidy (Jia *et al.,* 2016; Naylor & Van Deursen, 2016).

According to the results, the genes responsible for encoding the proteins involved in the first circuit proposed by Suescum and collaborators had a very low level of expression in colon cancer cell line HT29. Firstly, *PLK1* showed a low expression level which indicates that its interactions with CPC proteins

are affected. PLK1 protein can regulate AURKB indirectly by phosphorylating BIRC5 or FOXM1 (Li *et al.*, 2015). However, AURBK is fully activated upon binding and phosphorylating INCENP. INCENP is not only phosphorylated by AURKB but also by CDK1 at different phosphorylation sites, activating CPC. If this activation fails, centromeres wouldn't be able to localize during metaphase and relocalize in the midzone of the spindle during anaphase (Honda *et al.,* 2003). On the other hand, INCENP and AURKB phosphorylate PLK1 during mitosis to activate and recruit it to centromeres. In the centromeres, PLK1 contributes to the generation and stabilization of microtubules at the kinetochore (Goto *et al.,* 2006). Aditionally, AURKB corrects merotelic attachment between kinetochores and microtubules (Gregan *et al.,* 2011). Therefore, there is a signaling crosstalk between PLK-AURKB-INCENP and CDK1 that is crucial in the accurate segregation of chromosomes into two daughter cells. Thereupon, if *PLK1* and *INCENP* present low levels of expression, these mechanisms will fail. According to Goto and collaborators, analysis showed that when INCENP is inhibited, *AURKB* expression is reduced, and the protein is mislocalized. Also, it abolished the recruitment of PLK1 protein to kinetochores inhibiting its functionality (Carmena *et al.*, 2012; Goto *et al.,* 2006).

PLK1 has a great impact in the progression from metaphase to anaphase. It has the power to phosphorylate CDC20 protein and inhibit APC/C activation which initiates anaphase. When PLK1 has low concentrations or is inhibited, APC/C is not regulated, and it is activated before chromosomes are correctly aligned. According to Raab and collaborators, when PLK1 is inhibited cells show high expression of APC/C, with a decrease in mitotic deaths down to 6% and increased fraction of cells escaped mitosis and were able to survive up to 46%. These results suggest that the expression of APC/C and the inhibition of PLK1 act synergistically to weaken the spindle assembly checkpoint. It also promotes the premature and improper mitotic exit, leading to the formation of cell death resistant polyploid cell. If *PLK1* expression levels are low, the cell is exposed to premature mitotic exit, therefore CDK1 and cyclin B levels are very low. Cells exit mitosis more slowly when CDK1 and cyclin B levels are high because they are important parameters of mitotic exit timing. Considering Raab and collaborators observations, when APC/C is prematurely activated and PLK1 inhibited, there is almost a complete loss of the kinetochore-bound to AURKB and activity at these sites is lost. Accordingly, lack of PLK1 during metaphase and anaphase prevents a normal kinetochore assembly altering the core structure of kinetochores by APC/C expression (Raab *et al.,* 2018; Strebhardt *et al*., 2018).

CONCLUSION

Regarding the expression results, *PLK1* had no significant variation between the values in each cell line. Standard deviation results showed a wide variance value, which means in future experiments the biological replicability must change. However, the results show a trend where every target gene had a lower expression level in HT29 cell line, with respect to HEK293 cell line. HT29 portrayed the behavior of a cell that is entering its neoplastic transformation. Which means it has not developed every single

characteristic of a neoplastic cell. This could be a reason why these genes are not overexpressed but on the contrary, they are underexpressed. Somehow these cells are developing their genomic instability hallmark by contributing to chromosome missegregation by the downregulation of important genes in the process. Consequently, each protein will not be able to complete their function properly.

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ANNEXES

1. Excel document with each table of equations regarding expression average of each gene.
2. Excel document with table regarding qPCR results and ct values.