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Chemotherapeutic sensitivity and the expression of BIN1 in Human Diploid Fibroblasts expressing SV40 T-antigen

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By

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Abstract

Cisplatin is the chemotherapeutic of choice to treat several cancers, notably cervical and esophageal, which are commonly induced by the tumor virus HPV. Cisplatin's mechanism of action is to create intra-strand and inter-strand linkages between DNA; however some tumors have been shown to have developed resistance. It has been postulated that resistance to cisplatin can be linked to BIN1 expression levels or the isoforms created as the result of splicing by SRSF1. BIN1 (bridging integrator-1) is involved in many functions of the cell, but most importantly it regulates the cell cycle and apoptosis through its tumor suppressor characteristics.

To help elucidate the mechanisms involved in cancer induction by tumor viruses and help further chemotherapeutic treatment options, this research was undertaken to determine if cisplatin was effective against cells transformed with the Simian Virus 40 (SV40) T antigen and to determine if the mechanism of action is linked to BIN1 expression levels.

To accomplish this, human diploid fibroblast immortalize with telomerase [HDF(tert)] were transfected with a plasmid encoding the SV40 T antigen; and, two distinct clones were expanded [HDF(tert)+T Clone 1 and HDF(tert)+T Clone 2]. All cells were exposed to cisplatin and growth was assessed. It was found that cells expressing the oncoprotein were more sensitive to cisplatin than the parental cell line. To determine if the parental line resistance to cisplatin correlated to BIN1, expression level and isoform production was assessed.

This research found that there is no difference in the accumulated levels of BIN1 protein being expressed among the 3 cell lines as determined by Western blotting. However, RT-PCR revealed multiple isoforms being expressed. The predominant form in all cell lines appeared to be isoform 10 which correlates to the 456 base pair band and the 45 kDa protein band. Interestingly, a new, ninth unpublished isoform was isolated which was present in both the immortalized parental line [HDF(tert)] and the transfected cell lines [HDF(tert)+T Clone 1 and HDF(tert)+T Clone 2]. Overall this data indicated that BIN1 expression is not solely responsible for cisplatin effectiveness. T-antigen-oncoprotein-expressing-cells were killed up to 90%; yet, BIN1 levels were equal to that of the non-transformed line. Whereas the non-transformed cells exhibited 50% survival. It is important to determine the mechanism for cisplatin sensitivity in order to apply it to make cancer cells less resistant to treatment.

Introduction

To treat a wide variety of cancers, the chemotherapeutic known as cisplatin is administered to patients (Tanida et al., 2012). Over time cancer cells have developed mechanisms to become resistant to this treatment method (Tanida et al., 2012). Research has found that bridging integrator-1 (BIN1) is linked to establishing resistance to therapeutics, like cisplatin (Tanida et al., 2012). Around 60% of breast cancers have been recorded to be BIN1 null, thus abrogating its traditional tumor suppressor characteristics (Tanida et al., 2012). When the wild type form of BIN1 is reintroduced the cancer cells cease proliferating through the p53 and caspase-independent death pathways (Tanida et al., 2012).

Cisplatin works to kill cancer cells, which have lost the ability to regulate the cell cycle. To block DNA replication, cisplatin binds to DNA to create intra- and inter-strands breaks (Tanida et al., 2012). As a result, the cell has activated DNA damage pathways and Rad3-related protein (ATR) phosphorylates serine-15 on p53 (Tanida et al., 2012). In addition to ATR, the extracellular signal-related kinases (ERKs), within the MAP kinase pathway, also have the ability to phosphorylate serine-15 on p53 (Tanida et al., 2012). Apoptosis ensues when Bax and Bak cause the release of cytochrome c from the mitochondria leading to caspase 9 activation (Tanida et al., 2012).

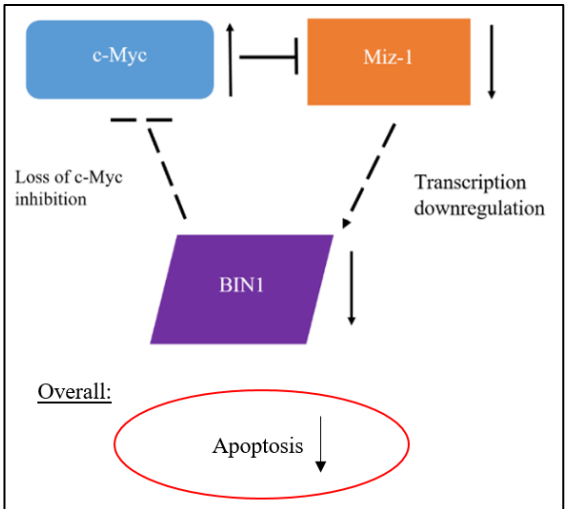


Figure 1. Cisplatin resistance mechanism involving BIN1. Lower levels of BIN1 expression leads to lower c-Myc regulation. With this comes less control over the cell cycle as c-Myc has the potential to promote the cell cycle when it should not resulting in a down regulation of Miz-1, a transcription factor promoting the expression of BIN1. All of this leads to less apoptosis, even when exposed to a chemotherapeutic.

Cells have been shown to obtain cisplatin resistance via three mechanisms: drug inhibition by molecules with thiols, efflux pumps, or increased amounts in DNA damage repair proteins (Tanida et al., 2012). Research has discovered a fourth resistance mechanism that involves prohibiting BIN1 from suppressing the proto-oncogene c-Myc, or blocking its binding to the DNA repair protein poly(ADP-ribose)polymerase (PARP) 1. Normally BIN1, as a tumor suppressor, binds to c-Myc to inhibit the transcriptional activator. Mechanistically, low levels of c-Myc lead to Miz-1 promoting the transcription of BIN1. This establishes a feedback loop resulting in low c-Myc expression levels (Tanida et al., 2012). If the BIN1 myc-binding domain (MBD) is non-functional or cells lose total BIN1expression, c-Myc binds to Miz-1 and it becomes a transcriptional repressor of BIN1, as seen in Figure 1 (Tanida et al., 2012). With low levels of BIN1, PARP1 is now free and tries to repair the DNA damage caused by cisplatin; thus, leading to resistance of the therapeutic and continued proliferation (Tanida et al., 2012). The BIN1 gene is not always deleted, MBD-mutants are created by alternative splicing.

BIN1 has a total of 20 exons that create a total of 8 known isoforms after alternative splicing (Figure 2). While ubiquitously expressed, muscle and neuronal cells typically have the highest amounts. In the cell, BIN1 has numerous functions including membrane recycling, cytoskeleton regulation, endocytosis, and DNA repair. The functional domains of BIN1 are displayed in Figure 2. The first 10 exons comprise the BIN1 N-Bar domain which allows for dimerization and creates curvature for the cell membrane. BIN1 has affinity to phosphoinositol in muscle cells due to the phosphoinositide (PI) binding motif in exon 11. Exons 13-16 comprise the CLAP domain providing BIN1 with the ability to bind to clathrin. The myc-binding domain (MBD) is encoded by exons 17 and 18; and, the last 2 exons are the SH3 domain implicated in interacting in other signaling pathways (Prokic et al., 2014).

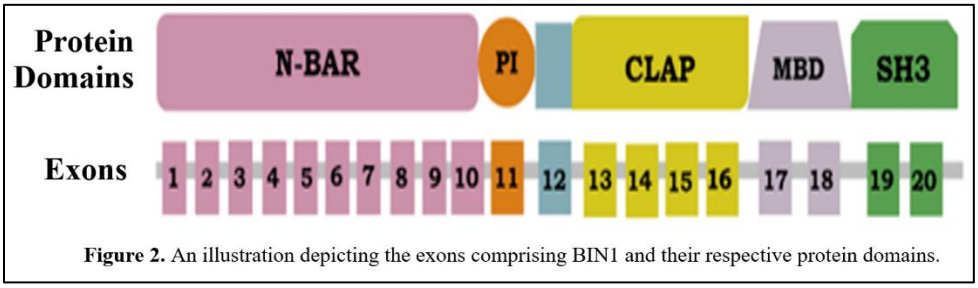


Figure 2. An illustration depicting the exons comprising BIN1 and their respective protein domains.

Alternative splicing of the heterogeneous nuclear RNA (hnRNA) result in multiple unique isoform of proteins (Anczukow et al., 2012). There is mounting evidence that alternative splicing plays a significant role in many cancers (Martinez-Montiel et al., 2018). There are over 15,000 alternative splicing events that have been connected with different aspects of cancer ranging from cell cycle proliferation to apoptosis or drug resistance (Martinez-Montiel et al., 2018). Overexpression of serine/arginine-rich splicing factor 1 (SRSF1) is linked to the alternative splicing of BIN1 resulting in isoforms lacking pro-apoptotic properties (Anczukow et al., 2012). As reported by Das et al., BIN1 lacking exon 17 no longer has the ability to bind c-Myc; in turn, c-Myc is able to encode for proteins to promote the cell cycle and in this under-regulated state is considered an oncogene (2012). Western blot analysis has shown increased amounts of SRSF1 lead to BIN1 missing exon 13 or 17 (Das et al., 2012). Figure 9 shows the published isoforms of BIN1.

With BIN1 reportedly playing a major role in sensitivity, it was important to determine this relationship in virally transformed cells that serve as a model for cervical cancer caused by the human papilloma virus (HPV). This research will investigate if BIN1 has a role in the cisplatin sensitivity levels of cell lines immortalized with telomerase and expressing the viral oncoprotein T antigen from SV40. To do so, the mRNA of different isoforms and protein expression levels of BIN1 will be looked at. A range of cisplatin treatments will be exposed to the cells to analyze growth rates. It is hypothesized that lower cisplatin sensitivity is due to a lack of BIN1. Determining if there is a relationship between BIN1 expression and overall cisplatin sensitivity is vital towards understanding how to develop alternative ways to treat resistant cancer cells.

Materials and Methods

Cell Culture. Human diploid fibroblast cells immortalized with telomerase [HDF(tert)] were graciously provided by K Rundell (Northwestern University). Two of those cell lines were transformed by the oncoprotein T antigen via the SV40 virus [HDF (tert) +T antigen Cl 1 and HDF (tert) +T antigen Cl 2]. Dulbecco's Modified Eagle Media (10% Fetal Bovine Serum, 7.5% Sodium Bicarbonate solution, and 1% Pen/Step mix) was used to maintain the cells and were grown in a 7% CO_2 incubator at 37°C.

Cisplatin Sensitivity. To determine cisplatin sensitivity, HDF(tert), HDF+T Cl 1, and HDF+T Cl 2 cells were seeded $1x10^5$ cells per well in plates containing 12, 20mm wells. After 24 hours, cisplatin was added in concentrations of 0.0 µg/ml, 0.5 µg/ml, 1.0 µg/ml, or 2 µg/ml

concentration of cisplatin in triplicate. Cells were incubated in cisplatin for 48 and 72 hours before harvesting for counting.

Western Blot. All cell lines were used to perform a Western blot for BIN1. A Bradford Assay was performed to obtain the protein concentrations and 100 µg of protein was added to an equal volume of 2X loading dye, heated to 100^{o}C for 5 minutes and then loaded into a 4-20% PAGE. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. The gel was transferred to nitrocellulose paper using an iBLOT machine (Invitrogen). Following transfer, the membrane was blocked with 8% non-fat milk in TBS-T (Tris-buffered saline, 0.1% Tween 20) for 1 hour at 4°C. The primary antibody (Invitrogen BIN1 Polyclonal Antibody Catalog #: PA5-82188) was added at a dilution of 1:200 and rocked overnight at 4°C. A 1X TBS-T solution was used to wash the membrane 4x. The membrane was moved to the SNAP machine for addition of the secondary antibody (Millipore). The membrane was incubated for 10 minutes with secondary antibody mouse anti-rabbit, (Santa Cruz Biotechnology sc-2357) then washed 4x with TBS-T. Chemiluminescent (ECL) chemical was added (Pierce™ ECL Western Blotting Substrate, Thermofisher) and the membrane was imaged using a GeneGnome (Syngene). BIN1 antibodies were stripped from the membrane using Restore buffer (Thermofisher) for a maximum of 10 minutes while rocking at 4^{o}C. TBS-T was used to rinse the membrane 3 times. The GAPDH primary antibody (Santa Cruz Biotechnology sc-365062) was diluted to 1:5,000 and added to the membrane and rocked overnight at 4°C. The identical procedure as stated above was followed for imaging the GAPDH loading control

RNA extraction and RT-PCR. RNA was purified from confluent cells using the RNeasy Plus Mini Kit (Qiagen™) according the manufacturer's procedures. cDNA for PCR was created using, the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) following manufacturer's protocol while using the maximum amount of RNA. Three sets of primers were used to amplify the 5', midsection, and 3' portions of BIN1 (Table 1). Hot start polymerase Taq was used for PCR and products were separated on a 1.8% agarose gel. DNA bands were excised and purified following the protocols from the QIAquick Gel Extraction Kit (Qiagen). Purified DNA was ligated into a pGEM-T Easy Vector using the pGEM-T Easy Vector System II (Promega). Alternatively, the PCR reaction was cleaned using the QIAquick Gel Extraction Kit (Qiagen) and an aliquot was placed into the overnight ligation. This plasmid was then inserted into competent DH5-alpha bacteria cells (Invitrogen). The bacteria cells (200 µl) were spread on Luria Bertani (LB) agar plates or 2X YT plates containing 50 µg/ml ampicillin, 50 µg of 20 mg/ml X-Gal and 100 µl of 100mM IPTG. Only white colonies were picked and grown in LB or 2X YT broth overnight containing 100 µg/ml of ampicillin. The High-Speed Plasmid Mini Kit (IBI Scientific) was used to extract DNA from the overnight cultures. Purified plasmid was digested with EcoRI to insure PCR fragment insertion and samples were sent to Eurofins Laboratories for DNA sequencing.

Primer	Forward	Reverse
5’	AAGGATCTCCGGACCTACCT	CACATTCATCTCCTCAAA
Midsection	TGAAGCCAAAATTGCCAAG	CTGAGATGGGGACTTG
3’	CCTGCAAAAGGGAACAAGAGC	TCATGGGACCCTCTCAGTGAAGTTC

Table 1: Sequences of BIN1 primer pairs. The 5’, midsection and 3’ primers correspond to exons 3-8, 6-12 and 12-20, respectively.

Results

The dose curve with varying amount of cisplatin, Figure 3, revealed similar results for both the 48 hour and 72 hour exposure time periods. The overall trend was that the more cisplatin, the less growth for both HDF(tert)+T Clone 1 and HDF(tert)+T Clone 2. However, for the HDF(tert) cells, there appeared to be some amount of resistance. Despite doubling the amount of cisplatin from 1 µg to 2 µg, there was a plateau of cell death. This suggests HDF(tert) cells are not as sensitive to cisplatin as the transformed cell lines expressing the oncoprotein T antigen. Further data analysis determined HDF(tert)+T are 48% more sensitive to cisplatin compared to the non-transformed HDF(tert) cells.

To determine if the cisplatin sensitivity was related to the amount of BIN1 expression in the cells a Western blot was performed. Western blots showed BIN1 was being translated into protein at relatively equal amounts in each cell line and GAPDH in the lower panels indicates that equal amount of protein was loaded (Figure 4). Surprisingly there was no lack of BIN1 as hypothesized; however, since specific isoforms of BIN1 could result in the same phenotype as a BIN1 null cell line, mRNA was examined.

When investigating the beginning 5’ and midsection portions of the BIN1 sequence in both untransformed and transformed cell lines, it was determined that all cells were missing exon 11 (Figure 5). Since the differences in the isoforms related to oncogenesis were expressed in the 3’ end, these segments were amplified. Gel electrophoresis of RT-PCR products showed the same banding pattern for each cell line’s 3’ end. Based on RT-PCR and gel electrophoresis, there appears to be approximately 6 BIN1 isoforms expressed in both HDF(tert) and HDF(tert)+T cell lines at varying intensities (Figure 6). Using calculations of exon size and investigating the reported isoforms, the identity of the bands could be predicted (Table 2). Table 2 lists all potential isoforms that could be found with their respective base pair size, which exons compose it, and kDa weight of the unmodified peptides. The RT-PCR products appeared to have amplified all bands expect for the potential neuronal; and, a new fragment was found between 300-400 base pairs (Figure 6B). The amplified products were bulk cloned into pGEMTeasy, transformed into DH5-alpha competent cells and colonies were selected, grown, and plasmid DNA prepared. DNA was sent to Eurofins Laboratories for sequencing and these results confirmed the presence of a novel isoform missing exons 13-19. One of the many replicate gels, Figure 7, shows the identical banding pattern with the presence of the same isoforms. It is worth noting that the

expression level of isoform 9 decreased for HDF(tert) and HDF(tert)+T Clone 1; however, this was not unusual. Through multiple RT-PCR experiments, we found variation in the expression level of isoforms, but the type of isoforms being expressed remained consistent. Figure 8 supports this by showing that all three cell lines were exhibiting higher than usual levels of isoform 9, but still maintain the typical banding pattern for the 3' end. Figure 9 depicts every reported isoform and the novel isoform found in this lab for comparison. According to the size of the bands, it appears the isoforms being expressed are isoform X12, isoform 9, isoform 10, and two unknown isoforms. DNA sequences confirmed the presence of isoform X12, 9, 10, and the novel Δ13-19 isoform.

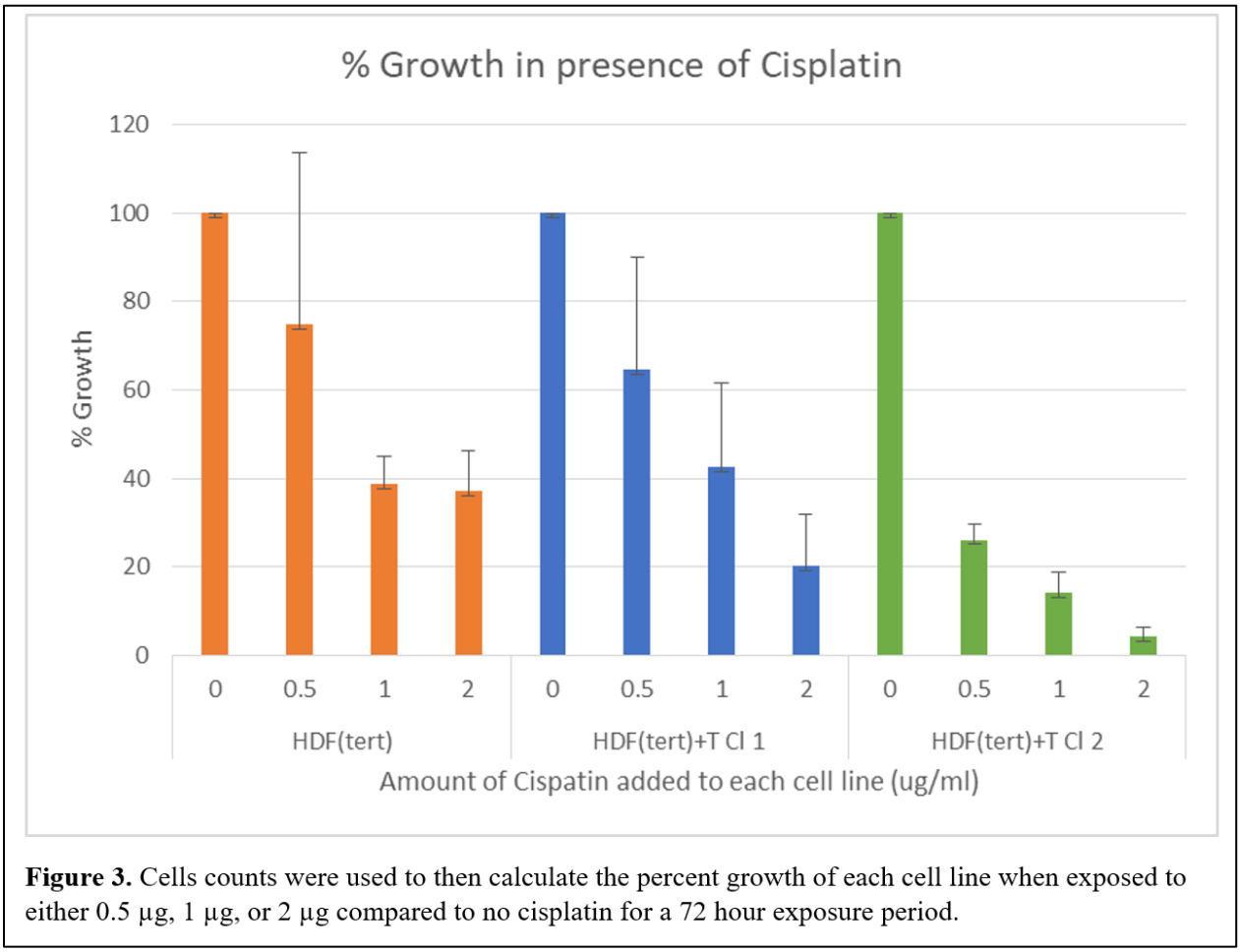


Figure 3. Cells counts were used to then calculate the percent growth of each cell line when exposed to either 0.5 µg, 1 µg, or 2 µg compared to no cisplatin for a 72 hour exposure period.

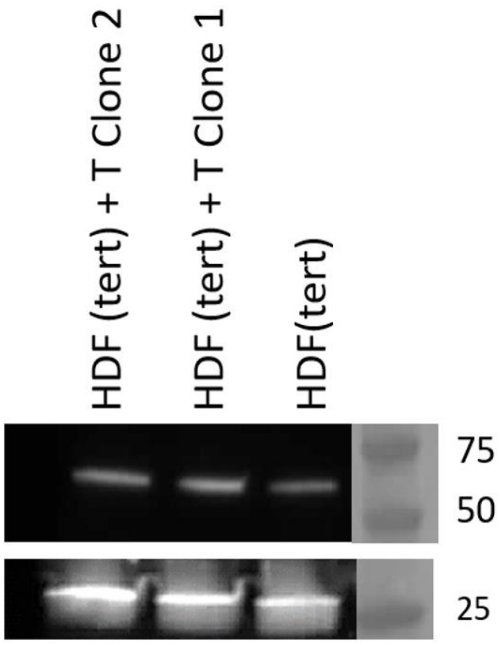


Figure 4. Western Blot analysis of BIN1 protein expression in HDF (tert), HDF (tert) + T Clone 1, HDF(tert) + Clone 2 between 50-75 kDa. GAPDH was probed around 25 kDa as a loading control.

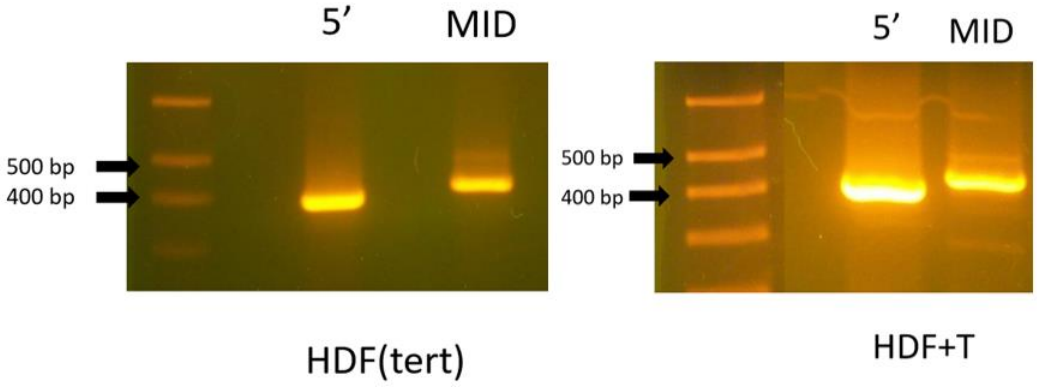
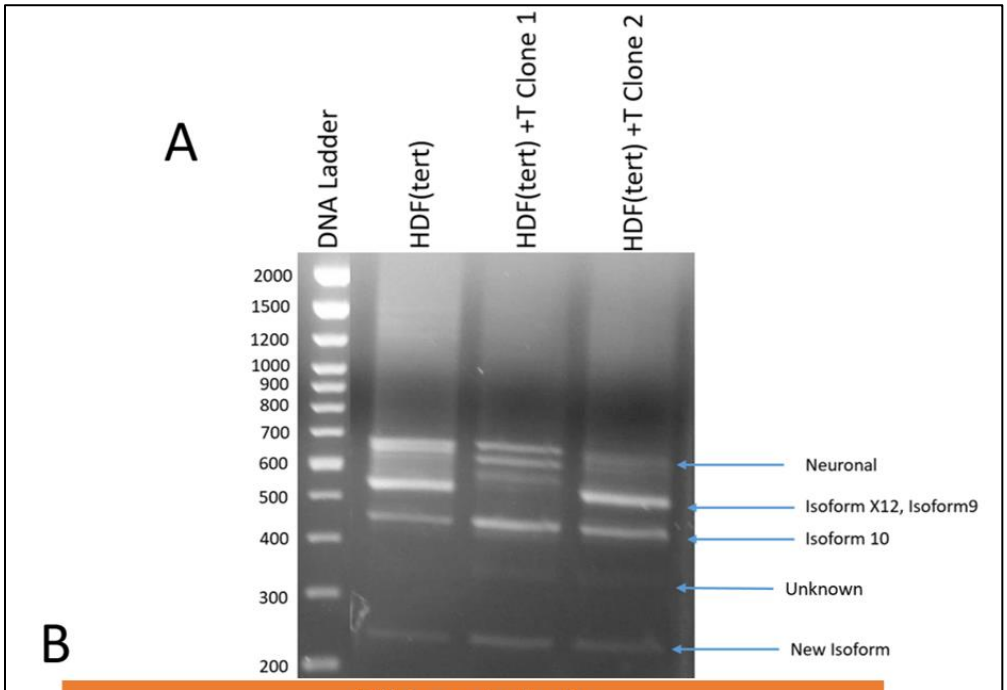


Figure 5. BIN1 products of the BIN1 5' and midsection from both HDF(tert) and HDF(tert)+T cells separated on a 1.8% agarose gel. 5' and midsection products were around the expected sizes of 409 bp and 476 bp, respectively.

Isoform	Missing Exons (starting from primer in exon 12)	Base Pairs (starting from primer in exon 12)	Full kDa size
Isoform 1	No exons missing	915 bp	65.23
Isoform 5	14 and 15	783 bp	57
Isoform 6	14-16	675 bp	53.02
Isoform 7	13-15	654 bp	52.25
Isoform 9	13-16	546 bp	48
Isoform 10	13-18	456 bp	45
Isoform X12	14-17	585 bp	49.72
Novel Isoform	13-19	243 bp	36.81

Table 2. Each isoform and their respective missing exons, base pair amount when using the primers, and entire full length kDa weight calculations.



DNA Sequencing Results	
Isoform 10	**456 base pairs**
Isoform 9	**546 base pairs**
Isoform X12	**585 base pairs**
New Isoform	**243 base pairs**

Figure 6. (A) Initial gel electrophoresis using newly synthesized primers from Eurofin Laboratories. The gel shows the presence of approximately 5 major bands representing both unknown and known isoforms. (B) DNA sequencing results once bands were sent out confirmed the presence of isoform X12, isoform 9, isoform 10, and a novel isoform. The neuronal and unknown bands between 300 bp and 400 bp have yet to be identified.

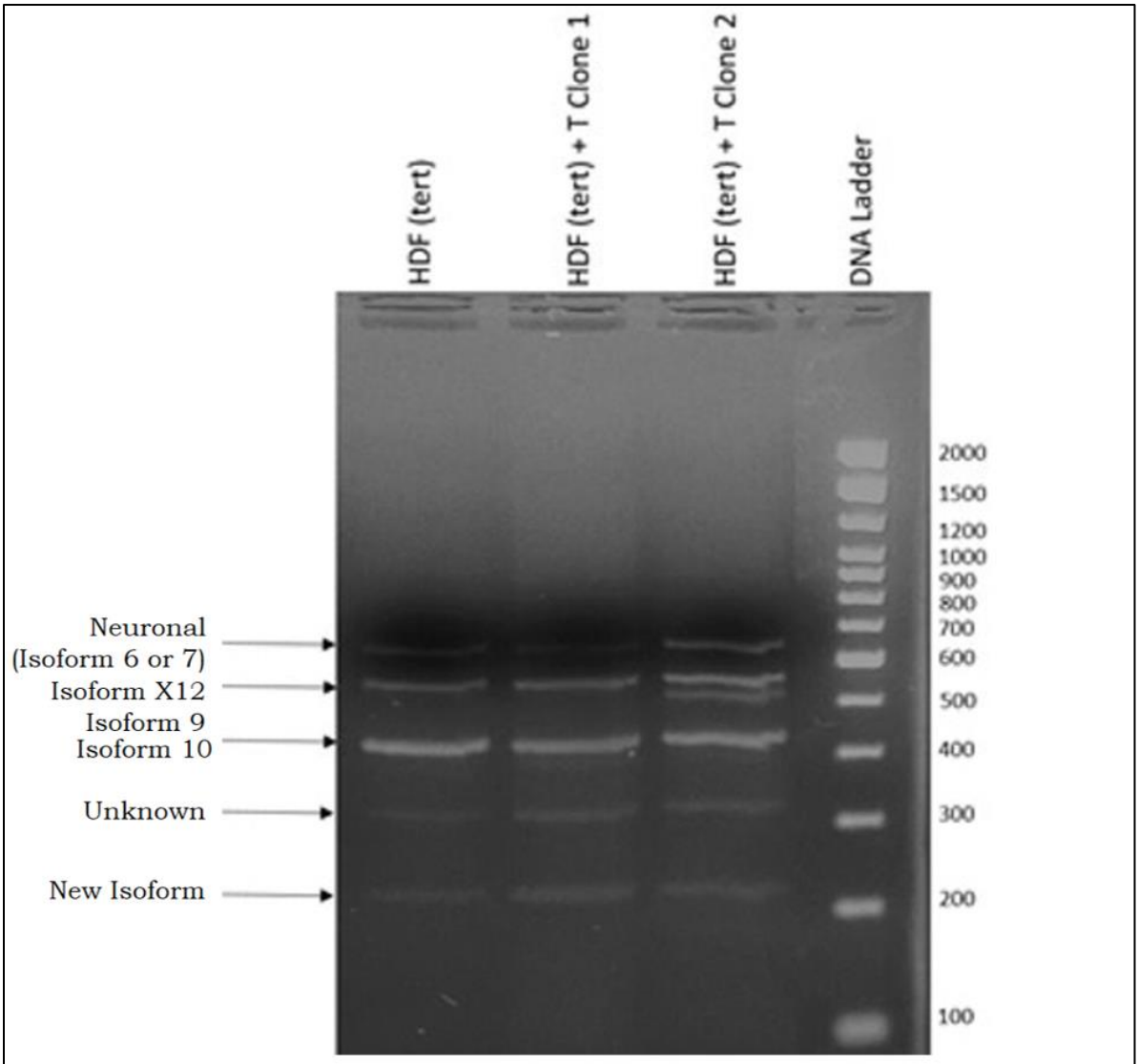


Figure 7. Gel electrophoresis of BIN1 PCR products in the three cell lines, HDF (tert), HDF (tert) + T Clone 1, and HDF (tert) + T Clone 2. The gel showed the presence of multiple isoforms of BIN1 mRNA being produced in the cell lines. The band identity was based on calculations and previous results.

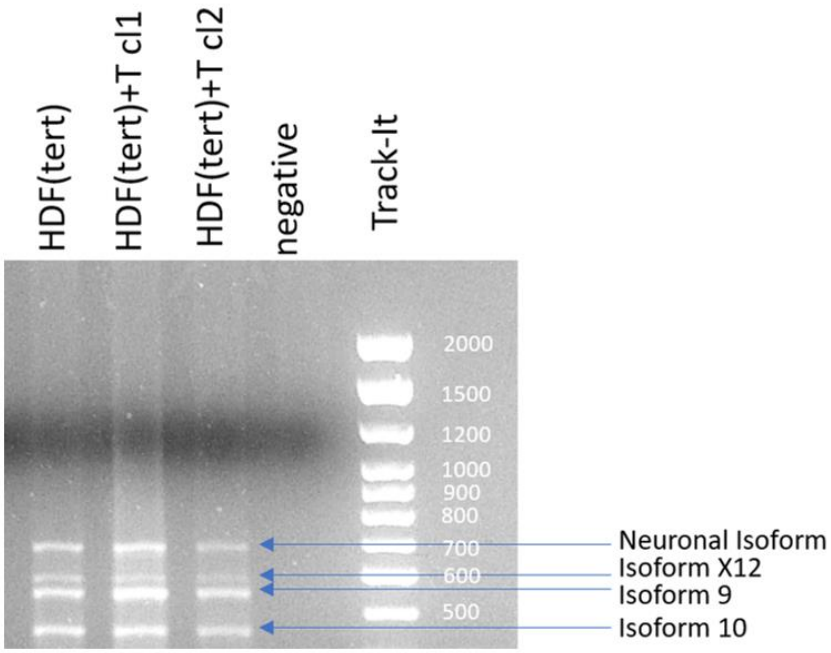


Figure 8. One of the many replicates confirming the same banding pattern exhibited in all three cell lines. However, this gel further confirms there is variance in the level of expression for each isoform when compared to other gels.

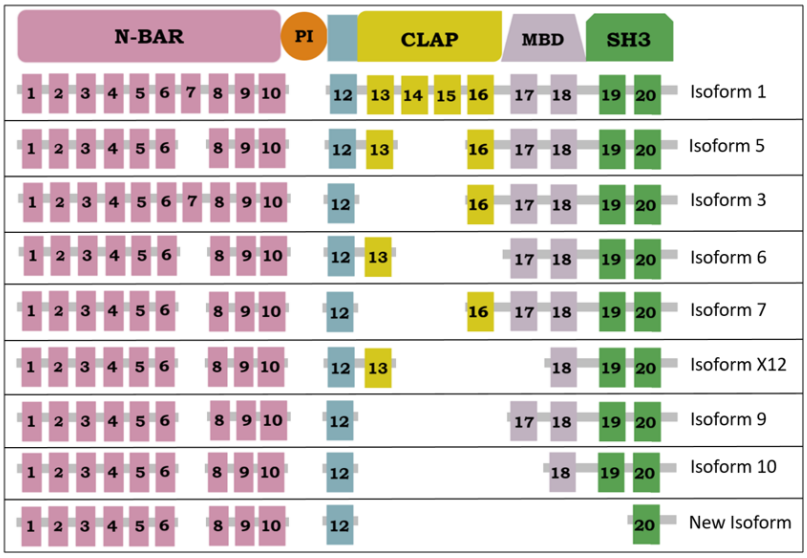


Figure 9. The reported isoforms along with the new isoform found in regards to which exons are present. Our cell lines are primarily expressing isoforms 9, 10, X12, and the new isoform. Isoforms 1 and 5 through 7 are neuronal isoforms and isoforms 9 and 10 are ubiquitously expressed.

Discussion

Determining the mechanism of chemotherapeutic sensitivity and resistance is germane to treating virally induced tumors. In this research study, the relationship between cisplatin sensitivity and BIN1 expression was investigated in immortalized human diploid fibroblasts transformed with the SV40 T antigen oncoprotein. In general it was found that the transformed cells were more sensitive to cisplatin than the immortalized controls, and we have shown that this is not due to an increased level of BIN1 expression. Therefore, the original hypothesis that HDF(tert) cells were more resistant to cisplatin because they had less BIN1 was rejected. Investigating further into whether there could be a difference in the isoforms of BIN1 being expressed yielded novel results.

Preliminary results have found no difference in the 5' and midsection of BIN1 between the HDF(tert) and the transformed cell line. As expected, exon 7 and exon 11 were missing from these two sections. Exon 11 was reportedly only found in muscle specific BIN1 isoforms; therefore, these results are not unusual because fibroblasts were used for this research.

Isoforms that were returned from the sequencing data yielded the isoforms matching bands at 243 bp (Δ13-19), isoform 10 at 456 bp, isoform 9 at 546 bp, and isoform X12 at 585 bp (Figure 6 and Figure 7). According to Prendergast et al. (2008) isoform 9 and isoform 10 are ubiquitously expressed; and, isoform X12 has been identified as a tumor isoform (Prendergast et al., 2008). Surprisingly the tumorous isoform X12 was found by sequencing in the HDF(tert) cell line. We hypothesized that this isoform is the result of forced telomerase expression for the purposes of immortalization (Wechsler-Reya, 1998). Since the new isoform lacks the MBD, it is believed to contribute the tumorigenesis of our cell lines based on isoform X12's structure and characterization.

There is a fifth band (approximately 660 bp) that appears in the gel, but did not come back in DNA sequencing results. Based on its high base pair amounts, it is predicted that the isoform is most likely isoform 6 or isoform 7 of the neuronal type (Prendergast et al., 2008). The band migrating between 300-400 base pairs has yet to be identified. The PCR band migrating between 300-400 base pairs could be predicted based on the exons and their respective base pair amounts as seen in Table 1. There are several possibilities, but the isoform could have exons 12, 19, and 20 to be a total of 345 base pairs. There were differences in the gels when it comes to expression level of each isoform, but the banding pattern remains consistent. Varying expression levels could be potentially be influenced by confluence level or any additional stress the cells could be experiencing.

Now knowing that there are multiple isoforms of BIN1 expressed in the cell lines, the Western can be further analyzed. Surprisingly there was only one band and it did not match the expected size of isoform 9, isoform 10, isoform X12, the neuronal isoform, or the novel isoform (see the last column in Table 2). The antibody used tagged the first 6 exons (amino acids 9-148) of BIN1, which according to preliminary research is present in all BIN1 isoforms expressed in our cell lines. A neuronal isoform could potentially represent the band that showed up, but another Western blot needs to be performed to determine if there were any post-translational

modifications that would have resulted in a heavier weight than expected. This will help determine what isoform is being produced as protein.

Overall, PCR and gel electrophoresis concluded that there is no significant difference in the types of isoforms being expressed among the cell lines. The sensitivity seen in the transformed cells cannot be due to a difference in BIN1 isoform. Despite this, the cell lines expressed a new isoform.

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