Understanding the Function of INI-1 Through Proteomics Analysis

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Abstract

INI-1 is a protein that is part of the SWF/SNF complex, and has been thought to maintain the cell cycle and mitotic spindle (Biegel 2006; Ginn and Gajjar 2012). Little research has been done on this protein so its function is still somewhat unknown along with its structure. The research completed in this study was done to gain more knowledge about the function of INI-1 based on the other proteins that are associated with it. The importance of this specific protein is because of development of Atypical Teratoid/ Rhabdiod Tumors or AT/RT in cells that lack INI-1 (Reddy 2005). AT/RT is a fatal pediatric brain cancer that has no proved treatments, and is diagnosed by the partial or complete deletion of chromosome 22q11.2 which is where the protein INI-1 is located. In this study I identify proteins that associate with INI-1 and discuss how this data helps to determine INI-1's function in the cell.

Keywords: AT/RT, INI-1, Proteomics

Introduction

Atypical Teratiod/ Rhabdiod Tumors were first recognized in 1987 and since then it has been discovered that these tumors are extremely malignant (Ginn and Gajjar 2012). AT/RT grows in the central nervous systems, mainly the posterior fossa, of children ages infant to about four years old, and today there is no cure for this cancer (Reddy 2005). This fatal cancer has unique genetic traits along with clinical structure. When immunohistochemical staining is done on these cells, AT/RT shows a partial deletion of chromosome 22q11.2 which is where the protein INI-1 is located (Ho *et al.* 2015; Reddy 2005). My hypothesis is that INI-1's absence is the reason for the formation of this deadly cancer. To determine if INI-1 is responsible for the cancer, its function needs to understood more. Some research has already been done, so we know that INI-1 is one of the ten proteins in SWI/SNF, which is a chromatic remodeling complex, and that INI-1 helps with cell cycle and mitotic spindle maintenance (Biegel 2006; Ginn and Gajjar 2012). These functions give some support for the formed hypothesis, but more information is needed to make a complete documentation about the function of INI-1. This proteomics study was done to identify proteins that are associated with INI-1.

Methods

Culturing HeLa Cells

For this study we used HeLa cells because of the ease of culturing them and because they have an undisturbed chromosome 22, so the INI-1 protein is there. For the study I did, I cultured a HeLa control group as well as an INI-1 Flag line. Both cells lines were made in the 1990's by the Kingston lab, and we purchased the lines from the National Cell Culture Center. To culture these cells, I used a 10 cm tissue-cultured-treated tissue culture dish. Growing the cells was done with 10mL of media, which was 500 mL of DMEM, 55 mL of FBS, and 5.5 mL of pen/strep antibiotics, and the plates were placed in a sterile incubator at 37° C and 5% CO_2. The cells are cultured until the HeLa cells are 80% or greater confluent in the culture dish. At this point the cells can be split into two plates. To split the cells, the old media is poured off, and the cells stuck to the bottom of the plate are washed with 5 mL of PBS which is aspirated off afterwards. To remove the live cells on the bottom of the plate, 2 mL of trypsin/EDTA are added and then the cells are incubated for about 5 minutes. Once the incubation period is done, the cells are removed from the adherent bottom by pipetting the trypsin/EDTA plus 8 mL of media up and down while squirting around the plate to remove all the cells. At this point, the cells can be added to a new plate and split evenly between two clean plates and 5 mL of media is added to each new plate and they are incubated. When ready the cells can be extracted from the plates using trypsin/EDTA and a cell scraper, pelleted down, and placed in a 2 mL tube and frozen at -80° C until used in the proteomics study.

Proteomics

The proteomics analysis in this study looks specifically at the proteins that associate with INI-1.

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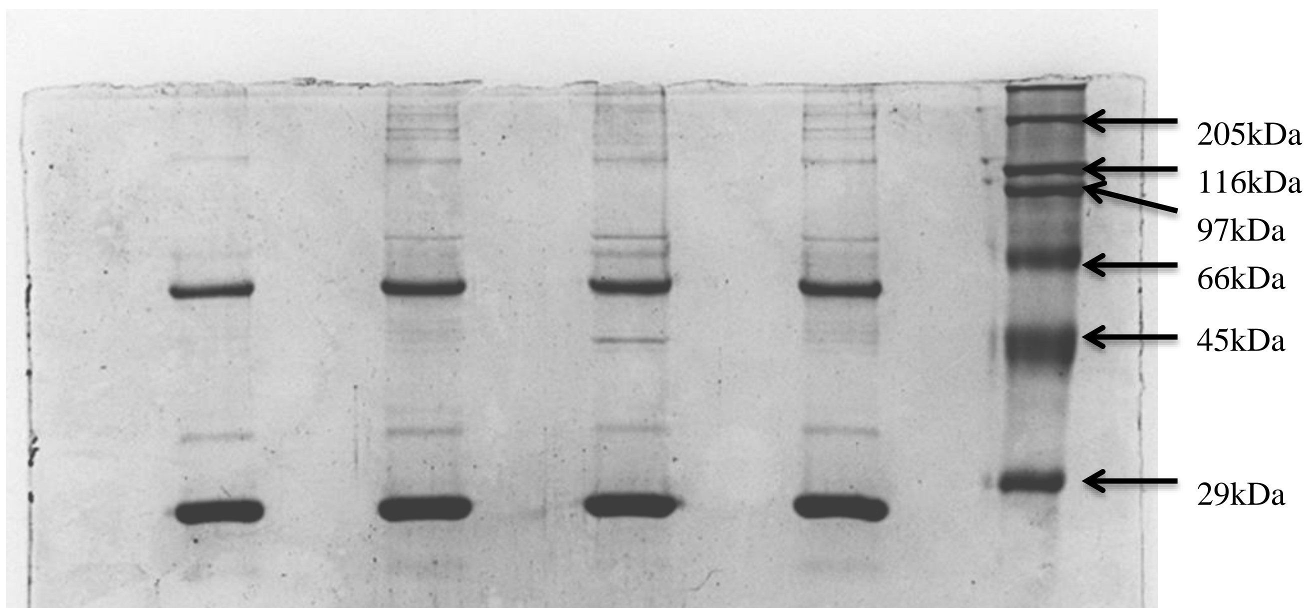

Figure 1 *- Hela Control Run (HCR) --- INI-1 FLAGGED Hela (IFH)--- High Molecular Weight Ladder (HMWL)*

To do this, an INI-1 Flag-tagged cell line was used to isolate INI-1 associated proteins. A wild-type HeLa cell line was used as a control. The first part of proteomics is to extract the protein from the cells, which was done with the freeze-thaw lysis method. The freeze-thaw method is done by resuspending the cell pellets in an extract buffer and freezing for 5 minutes at -80° C and then thawing completely and repeating this 3 times. Once this is complete the extract buffer plus cells are left on ice for 45 minutes. At this point the lysates are frozen at -80° C until proceeding to the next step. From this point all of the remaining steps are done at 4° C. The next step is to remove the soluble proteins from other cellular components. To do this the first thing is to centrifuge the tubes with the lysate at 13000 rpm for 10 minutes, and pull off the supernatant and place this in a new 1.5 mL tube. The pellets were saved in -80° C, and lysates were centrifuged again at 4000 rpm for 2 minutes. After this spin down, 2/3 of the total volume is pulled out from the center of the tube to avoid the invisible pellet at the bottom and floater at the top. This 2/3 of lysates contains the proteins that are put with 25 µL of washed FLAG M2 Resin and are nutated on the rocker for 2 to 5 hours. Following this step, 1 mL of wash buffer is added to the samples and inverted 10 times before being centrifuged for 2 minutes at 4000 rmp. The spin down pulls the resin to the bottom and the supernatant is pulled off. This is done 3 times with wash buffer and then twice more with proteomics buffer. It is extremely important to leave all the resin in the bottom of the tubes. The two samples are now washed and ready to be split: one tube for an SDS-PAGE gel and one for mass spectrometry. To split the samples, 100 µL of proteomics buffer is added to the samples and inverted. Then, 55 µL is pulled off from the bottom of the tube, spun down, the supernatant is removed so it is just the resin, and labeled for mass spectrometry. This is also done to the tube left for the SDS page gel. The mass spectrometry sample is saved in -80° C. The SDS page gel sample is mixed with loading dye and either placed in -20° C or immediately ran on the SDS page gel. To run the SDS-PAGE gel the samples are boiled for 5 minutes before being placed in the wells of a 12% SDS-PAGE gel and ran with a voltage of 120 V for about 90 minutes. After completing the run, the gel is placed in nano-pure water to destain before being placed in coomassie blue dye to reveal the bands of protein. These bands prove that all the steps done were successful and that mass spectrometry will be able to identify a lot of proteins. This process was done successfully twice and both tests were sent off to mass spectrometry. The SDS-PAGE gel in Figure 1 shows that there are proteins in both samples.

Mass Spectrometry

After the results from the SDS page gel, the other sample of HeLa lysates and INI-1 Flagged lysates are sent off to mass spectrometry. Dr. Steve Hartson in the Biochemistry & Molecular Biology Department at Oklahoma State University did the mass spectrometry study on the samples. The data produced from this study shows all of the proteins in each sample and the relative amounts. There were over 300 proteins that showed up in the study, but only about 150 of them were significant between

Table 1

Protein	T-Test (p-value) (p< . 05)	Biological Role
Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4	<.00010	Cytokinesis, cell shape, secretion, and capping
Isoform 2 of Filamins-A OS=Homo Sapiens GN=FLNA	.020	Promotes orthogonal branching of actin filaments and cell-cell contact
SWI/SNF complex subunit SMARCC1 OS=Homo Sapiens GN=SMARCC1 PE=1 SV=3	<.00010	Part of SWI/SNF, transcriptional activation, chromatin remodeling, part of npBAF complex
40S ribosomal protein S4, X isoform OS=Homo sapiens GN=RPS4X PE=1 SV=2	.0059	Translation, RNA binding, gene expression, viral life cycle
Isoform B of SWI/SNF related matrix associated actin dependent regulatory of chromatin subfamily B member	.00011	Core component of SWI/SNF, Cell proliferation and differentiation, inhibitor of tumor formation

samples. To farther analyze the data, I used a proteomics software called Scaffold 4. With this software, I was able to do a T-test on the data and get the p-value for each sample. Looking only at the low p-values and doing research on the website www.uniprot.org, I was able to determine the function of most of the proteins that associate with INI-1. In Table 1, I listed 5 of the most significant proteins based on a low p-value along with the proteins role in the cell.

Results

With the data from Dr. Hartson, there were over 300 proteins identified in the samples. Only about 150 of these proteins are prevalent in the study I am doing. For example, I overlooked all of the keratin, considering it was probably just a contamination from my skin cells. By looking at the results on the www.uniprot.org website, I was able to determine the function of multiple proteins. The proteins that I researched, due to their low p-value, revealed that most the proteins that associate with INI-1 have roles in a cell such as cell structure maintenance, translation, RNA binding, and/or cytokinesis. These results can give an idea to what INI-1's function is and give insight into how INI-1 inhibits tumor. A couple of the proteins that I focused on support my hypothesis, and they are listed in Table 1. The protein Isoform B of SWI/SNF related matrix associated actin dependent regulatory of chromatin subfamily B member is INI-1, and due to the flag-tagged HeLa cell line, it was expected to show up. Another significant protein is the 40S ribosomal protein s4, which is listed in Table 1; this protein is part of gene expression. With data like this, I can determine some of the roles INI-1 has in the cell.

Discussion

The proteins that I researched due to their low p-value revealed that most the proteins that associate with INI-1 have roles in a cell such as cell structure, translation, RNA binding, and/or cytokinesis. These results can give an idea as to what INI-1's function is and give insight into how INI-1 inhibits tumor. The protein Isoform B of SWI/SNF related matrix associated actin dependent regulatory of chromatin subfamily B member is INI-1, and due to the flag-tagged HeLa cell line, it was expected to show up. Another significant protein is the 40S ribosomal protein s4, which is listed in Table 1; this protein is part of gene expression. With data like this, I can determine some of the roles INI-1 has in the cell.

In the future, I will be getting AT/RT cells to culture and I will be studying the effects of re-expressing INI-1 in the cells. By using cellular proliferation, I will study the growth rates of the cancer cells with INI-1 re-expressed on a retro virus, different truncations of INI-1, and then a control set with just a retro virus. The hope with this study is that the re-expression of INI-1 in the AT/RT cells will show a slower growth rate than the control. This would support for the hypothesis that INI-1 is a tumor inhibitor.

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