The Expression of Ecotropic Virus Integration Site-1 in Seven Cancer Cell Lines

Abstract

The ecotropic virus integration site-1 (*EVI1*) gene is a transcriptional repressor implicated in the control of cell proliferation and frequently over-expressed in cancerous cells. I investigated the expression of this gene across seven cancer cell lines of varying morphologies. The tested lines included leukemia lines Kasumi-3, U937, MOLT-4, and CEM, breast cancer line MCF7, colorectal cancer line HT-29, and glioblastoma line M059K. Kasumi-3 and HT-29 are documented to have high *EVI1* expression. Protein concentrations were normalized with respect to actin using SDS-PAGE and Western blotting. Western blots for EVI1 showed expression of an unidentified protein with a molecular weight of 50-53kD in all lines except for Kasumi-3, which had no detectable protein expression. The intensities of these bands were measured and normalized with scaling factors determined from the Western blot for actin. The expression of *EVI1* may be below the detection threshold of this blotting system, making visualization of the protein difficult.

Focusing Question

Initial Question: Is over-expression of *EVI1* a common trait in cancerous cell lines?

Final Question: How does expression of *EVI1* vary among cancerous cell lines of different morphologies?

Rationale: Due to the lack of a true standard for over-expression, the experiment naturally tended toward comparing levels of expression rather than determining over- or under- expression. Additionally, the final focusing question reflects the complex nature of experimentation more accurately than the simplistic yes/no initial question.

Introduction

The process of cellular growth and replication is called the cell cycle. Proper development relies upon a complex system of checks and balances to prepare the cell before division and prevent unwanted growth. Cancer is characterized by out-of-control duplication of underdeveloped cells which lack these regulation mechanisms. Abnormal expression or mutation

of proteins leaves the cell incapable of arresting the cycle (American Cancer Society [ACS], 2010), and accumulation of these deleterious mutations can result in malignant growth. One third of women and one half of men in the United States will eventually develop cancer (ACS, 2010). Current research focuses on finding mutations and other traits unique to cancer cells to provide specific targets for treatment (ACS, 2009).

The ecotropic virus integration site-1 gene *(EVI1)* is found at the 3q26 locus (Liu, Chen, Ko, Fields, & Thompson, 2006) and expressed at very low levels in normal tissue (Soderholm, Kobayashi, Mathieu, Rowley & Nucifora, 1997). It is a transcriptional regulator with two DNA-binding zinc finger domains (Kurokawa *et al.,* 1998). Studies have shown that EVI1 interferes with the action of TGFβ, a transcriptional activator that signals growth arrest and initiates apoptosis. TGFβ activates Smad3, which signals the transcription of necessary genes (Liu *et al.* 2006; Kurokawa *et al.* 1998). Liu *et al*. showed that EVI1 suppresses TGFβ-mediated initiation of apoptosis through the recruiting of co-repressors to convert Smad3 from an activator to a repressor. In addition, EVI1 activates the anti-apoptotic pathway PI3K/AKT, which also interferes with TGFβ signaling by sequestering Smad3 (Liu *et al.* 2006; Conery *et al.,* 2004). Through these mechanisms, EVI1 encourages cell proliferation, interferes with differentiation (Morishita, Parganas, Matsugi, & Ihle, 1992; Goyama & Kurokawa 2010), and confers resistance to some chemotherapy drugs (Liu *et al.,* 2006).

EVI1 was first discovered in induced myeloid leukemia in mice (Nucifora, 1997). It has been established as a contributing factor in several cancers, including human myeloid leukemia (Langabeer *et al.,* 2006), colon cancer (Liu *et al.,* 2006) and ovarian cancer (Brooks *et al.,* 1996). It has been linked to pathogenesis and poor patient prognosis (Goyama & Kurokawa, 2010), and to chemotherapy resistance in tumor cells (Liu *et al.,* 2006). Other studies have shown that elevated expression of the isoform *MDS1/EVI1* (*MECOM*) and expression of the fusion gene *AML1/MDS1/EVI1* contribute to the generation of acute myeloid leukemia (AML) and poor patient prognosis (Senyuk *et al.,* 2002).

The expression level of a gene in a sample can be determined by the amount of its protein product that is detected. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) is a one-dimensional method of separating proteins based on molecular weight (Scheppler, Cassin, & Gambuer, 2000). To find the expression level of the EVI1 protein, I used

Western blots. A Western blot uses antibodies to detect a specific protein and quantify its expression level (Scheppler *et al.,* 2000). The expression of the protein can be compared among several samples based on the intensity of the florescent bands (Scheppler *et al.,* 2000). In order for these comparisons to be valid, the protein concentrations must be normalized with respect to a protein with confirmed, consistent expression in all samples. Once the same amount of this protein is detected in each sample, differences in band intensity for the target protein can be used to generate conclusions about gene and protein expression. I normalized with respect to actin, which is ubiquitously expressed in eukaryotic cells (Uniprot, 2011a).

I examined seven cancer cell lines to see if the over-expression of EVI1 is a widespread trait among different types of cancer or specific to a certain type. The cell lines were obtained from ATCC and are displayed in Table 1. Of these lines, Kasumi-3 and HT-29 have characteristically high EVI1 expression (ATCC, 2010). Based on previous findings and the known anti-apoptotic effect of EVI1, I hypothesized that all seven lines would display consistent, high levels of expression.

Materials and Methods

Cell Preparation: I cultured the seven cell lines in RPMI base media with fetal calf serum (FCS) and standard 1% penicillin streptomycin and 1% glutamine. Kasumi-3 required 20% FCS; all of the other cell lines grew well in 10%. I harvested the cell lines after two months of continuous growth and subculturing.

I determined cell viability for the suspended cell lines using a hemacytometer. Samples were diluted 1:1 with trypan blue to distinguish live and dead cells and counted to determine viability. I estimated adherent cell line viability by percent of the visible field adherent and percent of visible surface covered. MOLT-4, U937, CEM, and Kasumi-3 are suspended cell lines; M059K, MCF7, and HT-29 are adherent cells. Cell lines were harvested at >90% viability to ensure sufficient protein expression. After harvesting, the cells were frozen, and the cell lysate samples were prepared using a 1:1 ratio of phosphate-buffered saline with Tween (PBS-T) and Laemmli buffer. For all gels and Western blots, I used a 10% acrylamide gel.

Protein Detection: I used SDS-PAGE as a preliminary test to confirm the presence of protein in the samples. Using a standard volume for all cell lines, I performed a Western blot for

actin (β-actin mouse monoclonal primary antibody, sc-47778, Santa Cruz Biotechnology) and a series of Western blots for EVI1 (rabbit polyclonal primary antibody, ab28457, Abcam). I developed the blots using chemiluminescent techniques. The blots were incubated with a secondary antibody containing conjugated horseradish peroxidase, and developed using SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific). The horseradish peroxidase degrades the lumminol b in the developer, producing florescent bands. The blots were developed 1-3 minutes before photographing.

I determined the scaling factors for normalization from the actin blot using Gel Pro Analyzer. EVI1 blots were incubated with primary antibody overnight and with secondary antibody for two hours to compensate for the low constitutive expression. The intensities of these bands were also measured with Gel Pro Analyzer and normalized using the scaling factors from the actin blot. U937 was not used in this phase of testing.

Results

All lines were at 94% viability at harvesting, and all lines except Kasumi-3 had total cell counts of at least 71,000 (Table 2).

The Western blot for actin using 5uL of each cell line showed unequal actin expression (Figure 1). The scaling factors determined for normalization determined from this blot are displayed in Table 3. Kasumi-3 showed no protein expression, so it was not considered. The final EVI1 blot (Figure 2), was run with a standard concentration of 20uL. The protein shown is between 50 and 53kD and appears in all considered cell lines. The scaled intensities of this band for each cell line are displayed in Table 4. The highest expression of this protein was seen CEM and HT29, while the other cell lines showed similar, lower expression.

Conclusion

The results contradict my hypothesis and show no conclusive expression of *EVI1*. The final result, showing a protein of 50-53kD, does not match any known isoform of EVI1 (Uniprot, 2011b). Because of the low constitutive expression (Soderholm *et al.*, 1997), the EVI1 signal may have fallen below the detection threshold of this blotting system.

Discussion

Six isoforms of EVI1 have been documented, ranging in size from 117kD to 140kD. Other sources have evidence of isoforms with molecular masses of 88kD (Goyama & Kurokawa, 2010), and 180kD (Mitani, 2004). However, the protein shown in my analysis, running between 50 and 53kD, does not match any known isoforms.

Cross-reactivity occurs when an antibody recognizes an antigen that is common to more than one protein and binds to the region in a protein other than the target (Mayer, 2010). The Abcam antibody binds to residues 964-1013 on *EVI1* (Abcam, n.d.). Scans of protein databases show no sequence homology for this region except for other isotopes of *EVI1* (European Bioinformatics Institute, 2011; National Center for Biotechnology Information, 2011), indicating that the protein detected is not the result of cross-reactivity of the antibody.

My results contradict the findings of Liu *et al.*, (2006), Langabeer *et al.* (2006), and Brooks *et al.* (1996), who have found elevated expression in several types of cancer. EVI1 has been studied primarily in myeloid leukmias and documented as a dominant oncogene in this form of cancer (Goyama & Kurokawa, 2010). Over-expression has also been documented in HT-29 (Liu *et al.*, 2006) and metastatic breast cancer (Patel *et al.*, 2011). However, De Weer *et al.* (2006) demonstrated that EVI1 expression is normal in acute lymphoblastic leukemia (ALL), suggesting that is not a globally over-expressed protein. Of my tested lines, both CEM and U937 are derived from ALL.

Further blots are needed to confirm or refute the presence of the protein at 50-53kD. Testing with a more specific antibody may reveal this as nonspecific binding; if not, further testing is necessary to identify the protein. *EVI1* is known to be expressed at very low concentrations in normal cells (Soderholm *et al.* 1997), supporting my conclusion that the blotting system was not sensitive enough to detect the protein. No conclusive study of *EVI1* expression patterns in different types of cancers has been conducted. My study suggests that with further testing, *EVI1* or the unidentified protein found in my investigation may become a viable broad-spectrum therapeutic target for cancer treatment.

Inquiry Process

Student Inquiry and Research has taught me an immeasurable amount about independence and perseverance. Looking back through my journal, I can trace the evolution from depending almost completely on my advisor to dictate the next step to making most of my own decisions about the process. I learned to research and reason through questions, issues, and data in much more complicated ways than I have ever been exposed to in a science course. SIR allowed me to delve much deeper into the area of my interest and provided the chance to experience the true process of scientific research. In addition, I learned to reshape my questions to more complex, nuanced inquiries. My lab skills evolved from rudimentary to somewhat proficient at the tests required for my investigation, and at basic lab skills such as making solutions.

SIR has drastically changed my concept of research. I came in with very little knowledge about constructing a scientific investigation. Initially, I wanted to tackle much too broad of a topic. I had no concept of the amount of time it takes to generate even a small, insignificant result. Going through the process has revealed the less glamorous reality and given me a feel for what can be accomplished in a year. It has demonstrated, to a small degree, the amount of work that must be put into a project to obtain even a small result.

I chose an on-campus biology SIR due to difficulties finding an off-campus advisor. Although this was not the path I planned to take, I have no regrets. Being on-campus allowed me to build my lab skills and confidence with the research process, and to gain basic skills that I may not have been exposed to in a larger laboratory. I was responsible for the entire experiment, so I got the experience of working through each issue, and had the opportunity to work in the lab each day instead of once a week. This process has confirmed my interest in biological research. Although it is often difficult, I discovered that I truly enjoy the process of discovery, even when the results are nonexistent or difficult.

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Figures and Tables

Table 1. Cell lines and morphologies.

Catalog Number	Cell Line	Morphology
CRL-1582	MOLT-4	Lymphoblast; Acute lymphoblastic leukemia
CRL-1593.2	U-937	Monocyte; Histiocytic leuekmia
CCL-119	CCRF-CEM	Lymphoblast; Acute lymphoblastic leukemia
CRL-2725	Kasumi-3	Myeloblast; Acute myeloblastic leukemia
HTB-38	HT-29	Epithelial; Colorectal adenocarcinoma
HTB-22	MCF7	Epithelial; breast adenocarcinoma
CRL-2365	M059K	Fibroblast; Malignant glioblastoma

Cell lines were obtained from ATCC.

Table 2. Viability and cell count at harvesting.

Cell Line	Viability	Total Cell Count
MOLT-4	98%	93,000
U937	100%	85,000
Harvest 2	100%	394,000
CEM	94%	71,000
Harvest 2	100%	372,000
Kasumi-3	100%	36,000
HT29	N/A	N/A
MCF7	N/A	N/A
M059K	N/A	N/A

Suspended cell line viability and cell count were determined using a hemacytometer. Molt-4, U937, CEM, and Kasumi-3 are suspended cell lines. HT-29, MCF7, and M059K are adherent cell lines. Adherent cell line viability and total cell count could not be measured conclusively.

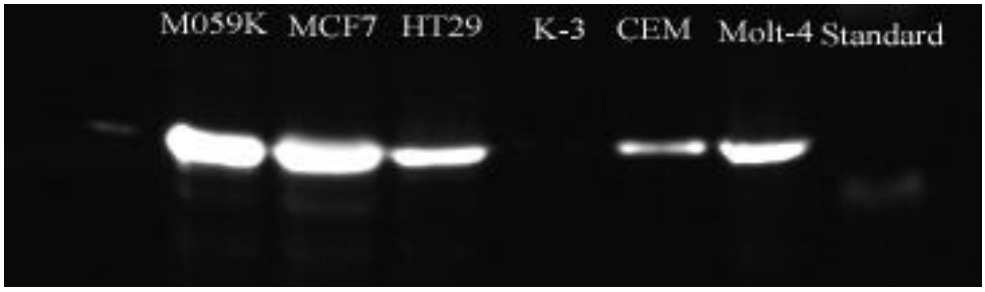

Figure 1. Western blot for actin. All samples were 5uL. Actin runs at 43kD. Using Gel Pro Analyzer, intensity of each band was measured and scaling factors were determined. U937 was not used. Kasumi-3 displayed no protein expression (lane 4), so it was not considered.

Table 3. Scaling factors

Cell Line	Scaling Factor
MOLT-4	1.117
CEM	3.984
HT-29	3.326
MCF7	1.074
M059K	1

Using Gel Pro Analyzer, the intensities of the bands from the actin blot were compared to generate scaling factors. M059K acted as the base.

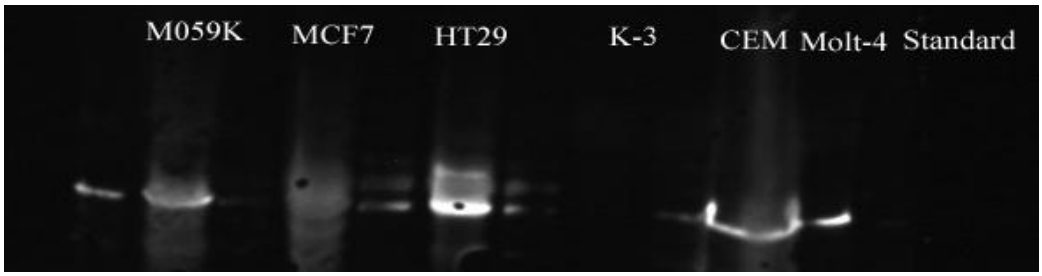

Figure 2. Western blot for EVI1. All samples were 20uL. The most prominent band ran between 50 and 53kD. The intensity of this band for each cell line was measured using Gel Pro Analyzer and normalized with the scaling factors. U937 was not included and Kasumi-3 showed no protein expression.

Table 4. Raw and scaled pixel counts comparing florescent band intensity of the unidentified protein at 50-53kD

Cell Line	Raw Numbers	Scaled Numbers
MOLT-4	42.376	47.33
CEM	39.478	157.3
HT29	44.457	147.9
MCF7	23.822	25.58
M059K	38.2	38.2

The raw intensity measurements were determined using Gel Pro Analyzer. The scaled numbers show the intensity of each band after normalization with the scaling factors.