INTERNSHIP ARTICLE

Mutations in Diaphyseal Medullary Stenosis-Malignant Fibrous Histiocytoma Related Gene MTAP Affects Expression of Splice Variants SV2 and SV5

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Abstract

Diaphyseal Medullary Stenosis- Malignant Fibrous Histiocytoma (DMS-MFH) is a rare autosomal dominant bone syndrome. Thirty-five percent of patients diagnosed with DMS-MFH are at risk of developing a bone sarcoma, malignant fibrous histiocytoma (MFH). Symptoms of this bone disease include bone infarctions, bone pain, leg weakness, and the development of early-onset cataracts. Recently, studies in our laboratory have shown that either of two inherited mutations, IVS8 (-2) A>G or Ex9 (+72) A>G in the MTAP gene locus, results in the development of DMS-MFH. These mutations specifically target the methylthioadenosine phosphorylase (MTAP) gene, located on chromosome 9p21.3. Our laboratory has now shown that the MTAP gene encodes six alternative splicing isoforms. The effect that the two disease-causing mutations have on MTAP splice variant 1 (SV1) and MTAP splice variant 4 (SV4) is complete skipping of exon 9. This project focuses specifically on two additional MTAP isoforms: SV2 and SV5. We chose to work with these two splice variants because they have similar ends and according to our results, one mutation coordinately regulates the expression of both variants. Intriguingly, loss of MTAP activity has been reported in a number of cancers, suggesting that these splice variants may play a role beyond those becoming known for this syndrome. DNA sequence and quantitative real-time PCR (qRT-PCR) demonstrated that only mutation IVS8 (-2) A>G was found to have an effect on the splicing of MTAP SV2 and SV5. In the presence of this mutation, the spliceosome skips the first nine bases of exon 9 as it binds to a weaker acceptor site. This new deleted splice variant was not present in samples containing the Ex9 (+72) A>G mutation. Since both mutations have similar general features and cause similar phenotypic effects, a genotype-phenotype correlation was established. According to our results, the two mutations do not affect the splice variants in the same way and thus, the deletion of the first nine bases of exon 9 may not be associated with the phenotype of the syndrome.

Introduction

Rare diseases are often ignored simply because of the mistaken belief that only a few affected individuals suffer. What many fail to understand is that collectively, these rare diseases affect a significant portion of the world's population. Diaphyseal Medullary Stenosis- Malignant Fibrous Histiocytoma (DMS-MFH) is a rare disorder with currently 6 families known to be affected. Those affected may suffer from debilitations, leg and arm weakness, pathologic fractures, irregularity in bone growth, and early onset cataracts. Using linkage analysis and positional gene cloning, our laboratory has mapped the disease locus to chromosome 9p21.3 and identified the disease-causing gene[1]. Given that mutations in this gene result in bone dysplasia and bone cancer, understanding the function of this gene should provide an understanding of its role in normal bone health and how its dysregulation can result in cancer. Ultimately, we hope that understanding the functions of this gene may provide insight into better bone health and the treatment of bone cancer.

The methylthioadenosine phosphorylase (MTAP) gene was linked to DMS-MFH after the region was narrowed down. Before the research taken on by Mount Sinai, the MTAP gene was believed to have eight coding exons[2]. Dr. Camacho-Vanegas et al .(2012)[2] found that the MTAP gene indeed contains three additional exons and can encode six different alternatively splice variants, in addition to the previously well-characterized wild type form (Figure 1). Novel terminal exons were found in all of the MTAP splice variants. As shown in Figure 1, some of the isoforms contain three terminal exons (9s, 10, 11) and others contain only one (9L). It was established that approximately 40 million years ago, two independent retroviruses integrated downstream of terminal exon 8 of MTAP and at some point, during primate evolution, became part of the gene[2]. The MTAP gene encodes an enzyme necessary in the salvage pathway of adenine and methionine[3]. Adenine is essential for the energy of the cell and DNA synthesis while methionine is critical in the process of protein synthesis. Therefore, mutations in the gene can potentially cause an irregularity in the salvage pathway and consequently, the production of adenine and methionine. The reduction of MTAP activity has been accounted for many other cancers, such as osteosarcoma, lung cancer, breast cancer, liver cancer, and other dire cancers[2].

Previous studies established that two heterozygous changes are associated with DMS-MFH. Five- hundred controls were then analyzed for polymorphism. None of the controls had the heterozygous changes, strongly suggesting that the changes are pathogenic mutations and not present in unaffected individuals[2]. Through computational analysis, using ESEfinder Release 3.0 program, the data suggested that these changes could potentially affect splicing of the exons. The intronic change at position IVS8 (-2) A>G was predicted to obliterate an acceptor splice site while the identical change at position Ex9 (+72) A>G potentially obliterates an Exonic Splicing Enhancer (ESE)[2] (figure 2).

The purpose of this project is to determine the effect of IVS8 (-2) A>G and Ex9 (+72) A>G mutations on two of the novel splice variants: MTAP SV2 and SV5. Because IVS8 (-2) A>G and Ex9 (+72) A>G are both splicing mutations, we hypothesized that the effect of these two mutations on MTAP

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SV2 and SV5, in which exon 9 is the last exon of the variant, will be to generate isoforms that have a partial or a complete deletion in exon 9. The spliceosome will not recognize the splicing acceptor site, resulting in the activation of a cryptic splice site.

When the mutations target the bases and change them from adenine to guanine, the spliceosome searches for a weaker splicing signal. Normally, the spliceosome splices the strong site. Once the base is mutated, the spliceosome does not recognize the signal and searches for a similar complimentary sequence, known as the weak acceptor site. Previous experiments have demonstrated that both mutations cause complete skipping of exon 9 in MTAP SV1 and SV4[2]. After extensive studies on the two splice variants, results confirmed that the effects of Ex9 (+72) A>G and IVS8 (-2) A>G mutations on MTAP SV1 and SV4 are the same[2].

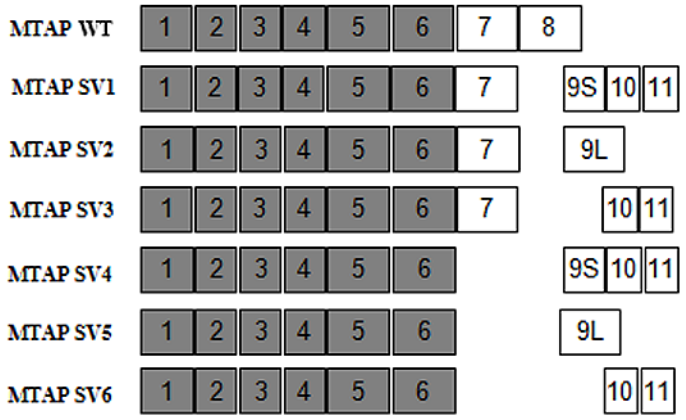

Figure 1. MTAP Splice Variants. There are a total of six known splice variants of the MTAP gene. The difference between exon 9s and exon 9L is their length. Exon 9s is 103 nucleotides long while exon 9L is 192 nucleotides long. (Figure from Camacho-Vanegas et al.2012)[2]

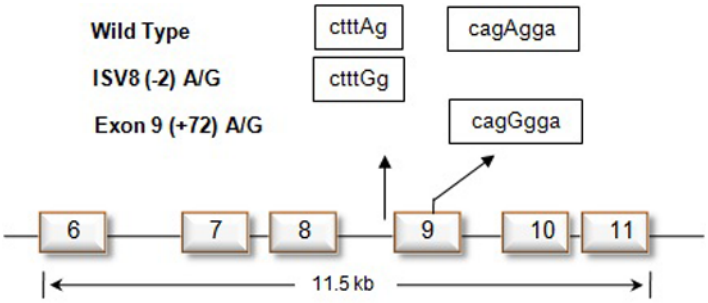

Figure 2. Sites of Mutation. IVS8 (-2) A>G mutation targets the ctttAg sequence in intron 8, replacing a single adenine base to guanine. Similarly, Ex9 (+72) A>G mutation targets the cagAgga sequence in exon 9, causing a point mutation (adenine to guanine). (Figure from Camacho-Vanegas et al. 2012)[2]

Materials and Methods

Previously, our lab generated three MTAP minigene constructs from exon 6 - exon 11 of the MTAP gene that included either the control, IVS8 (-2) A>G or Exon 9 (+72) A>G (Camacho- Vanegas et al.2012)[2]. These constructs were then transiently transfected into MCF7 cell lines in order to generate RNA transcripts for analysis. The isolated mRNAs were then reverse transcribed into cDNA, and each variant was amplified using forward primer Exon6F: 5' GACAGATTATGACTGCTGGA 3' and reverse primer Exon9Rev 5' GTTGTTGGAAGCAGTATCCAG 3'. After that, products were separated on the basis of size by gel electrophoresis, bands containing the correctly sized amplicons were cut from the gel and purified, and isolated nucleic acids were cloned and subsequently sequenced. qRT-PCR primers for each variant were then designed and lastly, the levels of each variant were quantified. MCF-7 breast cancer cells are functional in vitro models that were used for minigene transfection.Because these cells are MTAP-deficient, the extracted mRNA should come from only the minigenes. The following vectors were used: Minigene 1- pcDNA 3.1 (MTAP-Control), Minigene 2- pcDNA 3.1 (MTAP-IVS8 (-2) A>G), Minigene 3- pcDNA 3.1 (MTAP-Ex9 (+72) A>G). Dr. Camacho-Vanegas et al. (2012)[2] previously published the vectors that were used for this work. About 10 x 105 MCF-7 cells were plated in each well of a 12-well plate. The minigenes were transfected in triplicates using lipofectamine 2000 when the cells were ~70% confluent. Specifically, 1 ug of plasmid and 4.2 ul of lipofectamine was diluted in serum-free medium and put in each well. After 24 hours, cells were collected for mRNA extraction. RNA was extracted by following the given protocol of the RNeasy Mini Kit (Qiagen). After the elution of RNA, the nucleic acid was quantified using the Thermo Scientific Nanodrop 1000. 500 ng concentration of RNA was used. To reverse transcribe the given RNA samples into cDNA, we used the iScript cDNA synthesis kit. The final mix of each reaction included Buffer 5x, RT Enzyme, 500 ng of RNA and H20 to complete a final volume of 20ul. PCR was preformed twice using two different procedures: one with PCR Master Mix (Promega) (containing dNTPs, 10X Buffer, MgCl2 and Taq) and the other using the AmpliTaq Gold kit (Invitrogen) and adding each reagent separately. A total volume of 50ul was used for the PCR. The PCR reactions were prepared as follows: After PCR, 6x loading dye is added to each cDNA sample and then loaded into a 2% agarose gel (2 grams of agarose in 100 mL of TBE buffer) that is already mixed with 5 ul of ethidium bromide. The 1 Kb Plus DNA Ladder (Invitrogen) was loaded in the first and the last wells of the gel. The gel ran for 2 hours at 150 volts, which was enough to separate the bands. After gel electrophoresis, the appropriately sized bands were cut out and specifically categorized; upper bands were labeled as 'a' and lower bands were labeled as 'b' (illustrated in Figure 3). The Qiaquick Gel Extraction Kit (Qiagen) was used and its standard protocol was followed. Once the DNA was eluted, the nucleic acid was quantified using the Thermo Scientific Nanodrop 1000. 50 ng of eluted DNA was then ligated. We used the PCR TOPO Cloning Kit (Invitrogen) and followed its given protocol. 1 ul of ligation and 15 ul of Top10 competent cells were pipetted into six new 1.5 ml centrifuge tubes. The tubes were then held in ice for 30 minutes and after, were put in a 42*C incubator for 30 seconds. S.O.C medium was poured into each centrifuge tube and then put in the shaker at 37*C for one hour. After one hour, cells were plated into ampicillin petri dishes because vector pCR 4- TOPO carries the ampicillin resistance gene. The petri dish cultures were then incubated at 37*C overnight to allow colonies to grow. The following day, colonies were picked from all six samples and then individually put into a 15 ml tubes. Each tube consisted of 2 ml of LB, 2 ul of ampicillin (50ug/ul)

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and a single colony. The tubes were then put in the shaker machine at 200 rpm and 37*C overnight. A minimum of 20 colonies were picked for each band. The LB and bacteria solution was centrifuged after it was dispensed into a 1.5 ml centrifuge tube. Subsequent to centrifuging the tubes, the standard protocol for the Qiaprep Spin Miniprep Kit (Qiagen) was used for the purification of plasmid DNA. 0.2 ul of enzyme ECORI was added to the samples in order to have the insert released. 2 ul of Buffer 10x was poured into each of the tubes as well as 15.80 ul of sterile water. 2 ul of DNA, from the miniprep, was added to each tube as it was then placed in the incubator at 37*C overnight. The digestions that had two bands in the gel electrophoresis were successful in showing both the linearized vector and the insert. Once we verified that the minipreps contained inserts, the samples were then prepared for sequencing. At least 10 miniprep samples were sequenced for each band. The samples were sent for sequencing and then analyzed and aligned with the help of Sequencher v3.0 software application (Gene Codes Corporation). Each sample was compared with the control exons: 6, 7, and 9 of the MTAP gene. The chromatogram of the samples was traced and examined for any unusual patterns. We designed qRT- PCR primers for each splice variant. Each set of primers was tested for linearity, sensitivity, and specificity. Sequences for the specific primers are as follows: SV2DelForward Primer CATAACCTGAAGTTCCAGATGATT, SV5 DelForward Primer CGAGGAAGCATTCCAGATGATC, and SV2 / SV5 Reverse Primer GGCAGGATTTCACCTCTGCCA. Del represents the deletion of the first nine bases of exon 9. The dilution series of cDNA (Figure 5 (x-axis of line graph)-1: 1/10, 2: 1/100, 3 :1/1000, 4: 1/10,000, 5: 1/100,000) tests the linearity of the primers. The aim is to get a line closest to the regression coefficient approaching 1, avoiding contamination. The specificity of a primer is to determine what the primer is binding to. Good specificity means that the primer binds to only the selected sequences and nothing else. The sensitivity of a primer is how well the primers can detect the needed sequences. Once the primers passed all of the requirements, we quantified the level of each splice variant using them.

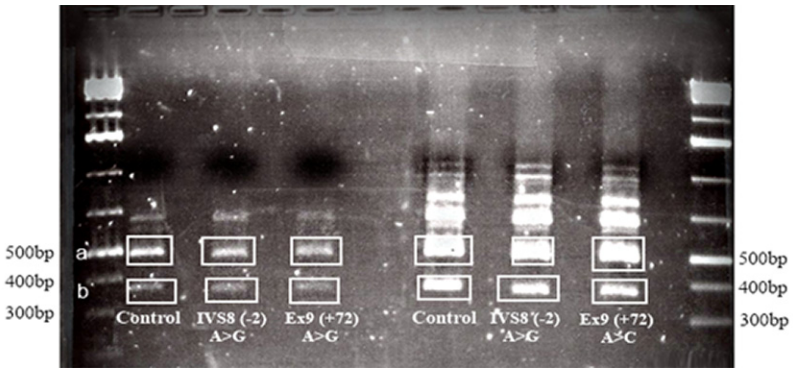

Figure 3. The figure above is a gel-imaging picture of three distinct samples that are labeled control, IVS8 (-2) A>G mutation, and Ex9 (+72) A>G mutation. The three samples on the left were PCRed using the AmpliTaq Gold kit, while the three samples on the right were with PCR Master Mix (Promega).

Results

According to figures 4a and 4b, the space that is filled with gap marks between exon 7 and exon 9 is a portion of the exon 9 sequence that the spliceosome skips in order to find its complementary splice site. The deletion of the first nine bases of exon 9 is found in samples that are derived from the IVS8 (-2) A>G mutation. However, such a deletion is not seen in sequences derived from the control and the Ex9 (+72) A>G mutation. The illustrated samples are only from the first experiment. Two more experiments were thereafter conducted with another set of samples to confirm the results.

According to our results, the weaker splicing site is after the first nine nucleotides of exon 9. Only mutation IVS8 (-2) A>G affected the genetic sequence of the splice variants. Mutation Ex9 (+72) A>G, however, produced no visible changes in the sequence. Table 1 highlights the number of colonies analyzed by DNA sequencing and the number of splice variants that have the deletion in the first nine bases of exon 9. In RT-PCR, the forward primer attaches to the beginning of exon 6 while the reverse primer attaches to the exon 9 end.

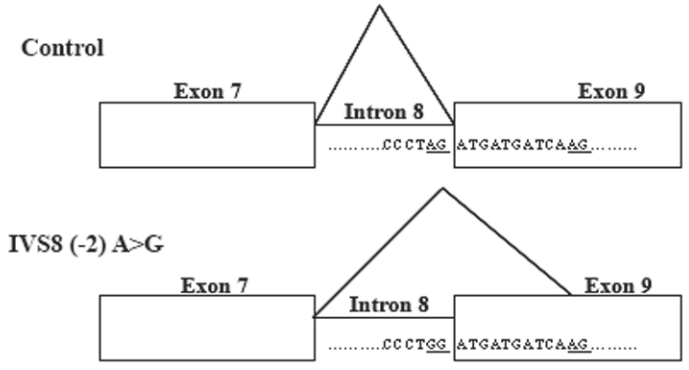

Figure 4a. Change of Acceptor Site. The figure above is an example of the control sample and the IVS8 (-2) A>G sample splice sites for MTAP SV2. With a base change from AG to GG in the splice acceptor site, the spliceosome continues to scan for another AG sequence to splice.

The four experiments shown in Figure 5 all have very good linearity with a regression coefficient approaching 1. The results were normalized by averaging the values of beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes. The first two experiments are testing the levels of MTAP SV2-deleted while the following two are testing the levels of MTAP SV5-deleted. According to Figure 5, the splice variants SV2-deleted and SV5-deleted were overexpressed when compared to normal only in cells transfected with the construct containing the IVS8 (-2) A>G mutation. SV2-deleted and SV5-deleted variants, however, were not present in cells transfected with the normal and +72 constructs.

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Discussion

Based on the results, only mutation IVS8 (-2) A>G affected the splicing of MTAP SV2 and SV5. This mutation was the source of the first nine nucleotide deletion of exon 9 in MTAP SV2 and MTAP SV5. In agreement with our hypothesis, the spliceosome looked for a weaker signal to splice and recognized the following AG sequence in exon 9. Figure 4a illustrates the control (ctttAg) and the mutated (ctttGg) splice acceptor site. With one mutation-affected nucleotide change, the spliceosome did not recognize the acceptor splice site and continued scanning to find a different site with the corresponding AG sequence. According to Figure 4b, the spliceosome found the complementary AG pair and spliced off the first nine bases of exon 9.

Based on the data in Table 1, IVS8 (-2) A>G mutation caused the deletion of the first nine bases of Exon 9 in SV2 and SV5 in 84% of the sequenced minipreps. We determined that Ex9 (+72) A>G mutation does not affect the splice variants because the deletion was not present in the miniprep sequences with Ex9 (+72) A>G. Due to a small pool of patients diagnosed with DMS-MFH, it is difficult to give a forward-looking diagnostic. However, a genotype-phenotype correlation was confirmed by previous research in the DMS-MFH project and was used as an agent in determining the mutations that are important in the phenotype of the syndrome. Based on this correlation, the deletion of the nine bases does not affect the phenotype because in order to correlate with the syndrome, both mutations should produce the same genetic effect, leading to similar symptoms. The genotype-phenotype correlation is only one way of analyzing our results. Another way is taking into account protein. The deletion of the first nine nucleotides of exon 9 may very well be significant. Nine bases are three codons, indicating that the reading frame is conserved. The three codons make up three amino acids; therefore, the IVS8 (-2) A>G mutation causes a deletion of the first three amino acids of exon 9. We can do in vitro experiments to test if the protein can be translated and functional. To see if such a deletion of amino acids affects the phenotype of the syndrome, we must first discover a specific phenotypic quality that is exclusive to only the IVS8 (-2) A>G mutation. It is only then that we can correlate the deletion of the three amino acids with a specific symptom that is just found in patients with the ISV8 (-2) A>G mutation.

In this project, we used MCF-7 breast cancer cell lines for transfection. MCF-7 breast cancer cells may not express some of the tissue-specific splicing factors that are in bone osteosarcoma cells. In the future, we can avoid negative results by transfecting with an osterosarcoma cell line. We can also analyze why and how deregulation of the levels of MTAP splice variants is involved in carcinogenesis. In order to do so, we will take different tumors and follow a similar methodology by measuring the expression of the splice variants via qRT-PCR. We will test the presence of the deleted variants in the various tumors and determine how overexpression or underexpression of variants correlates with protein expression and ultimately the phenotype of DMS-MFH.

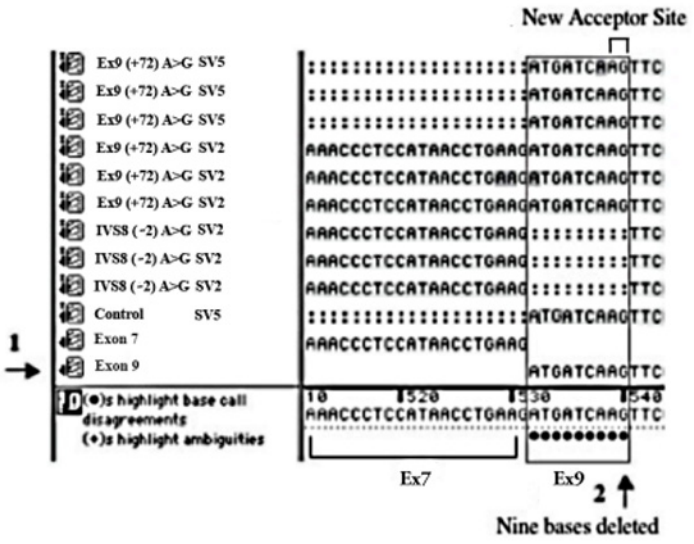

Figure 4b. Sanger Sequencing. The sequences of the samples were trimmed to avoid poor quality data, assembled (5' to 3'), and analyzed via the Sequencher v3.0 software application (Gene Codes Corporation). The exons of each sample were aligned with the exons of the controls. Arrow #1 points to the sequence of exon 9 that was used as a template to determine the location of the deletion. Arrow #2 points to the deleted region in samples with the IVS8 (-2) A>G mutation.

Table 1. Sequencing Analysis of SV2 and SV5 Expression in Cells with Each of the Minigene Constructs.

	TOTAL # OF COLONIES SEQUENCED
Control	11
IVS8 (-2) A>G	12
EX9 (+72) A>G	11

a-upper band
b-lower band

					PERCENTAGE
EXP. 1 -7.29.11					
COLONIES	SEQUENCED	SV2	SV5	(+9) BASE DEL EX9	
Control a	0				0%
Control b	1		1		100%
IVS8 (-2) a	3		3	3	100%
IVS8 (-2) b	3	3		3	100%
EX9 (+72) a	3		3		0%
EX9 (+72) b	3	3			0%
EXP. 2 - 8.4.11					
COLONIES	SEQUENCED	SV2	SV5	(+9) BASE DEL EX9	
Control a	3		3		0%
Control b	3	2	1		0%
IVS8 (-2) a	3		3	3	100%
IVS8 (-2) b	3	3		1	33.33%
EX9 (+72) a	3				0%
EX9 (+72) b	2	2			0%
EXP. 3- 8-12-11					
COLONIES	SEQUENCED	SV2	SV5	(+9) BASE DEL EX9	
Control a	2		2		0%
Control b	2	2			0%

TOTAL					
COLONIES	SEQUENCED	SV2	SV5	(+9) BASE DEL EX9	
Control	11	4	7		0%
IVS8 (-2) A>G	12	6	6	10	83.33%
EX9 (+72) A>G	11	5	6		0%

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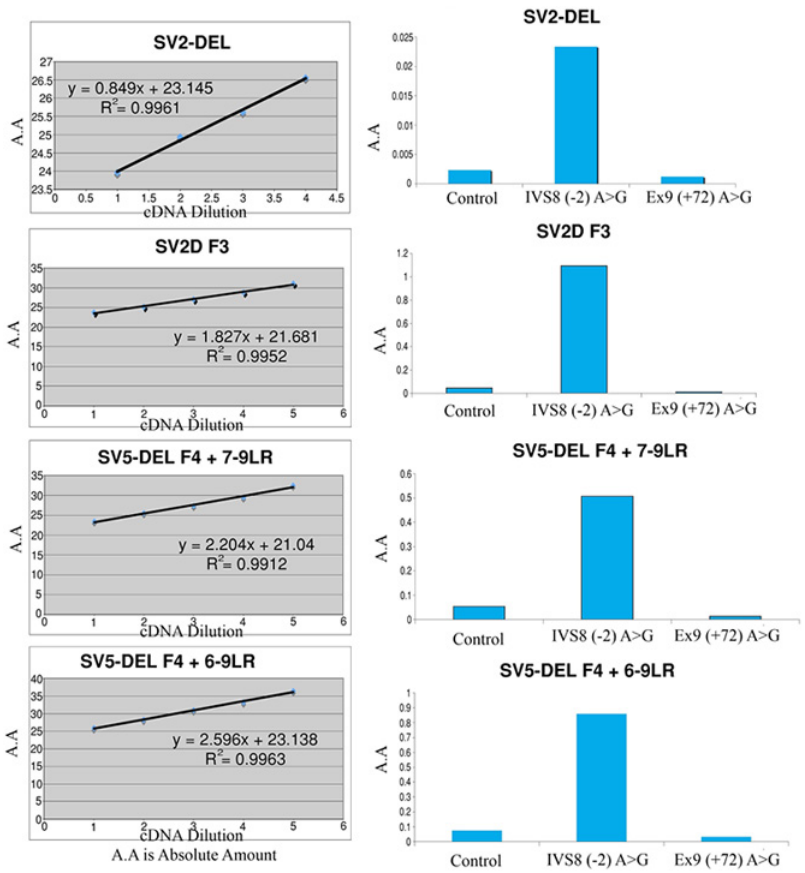

Figure 5. Quantitative RT-PCR Analysis of MTAP SV2-deleted and SV5-deleted Expression in Cells with Each of the Minigene Constructs. Using specific primers, I quantified the levels of MTAP SV2, SV5, SV2-deleted, and SV5-deleted by qRT-PCR. SV2- deleted and SV5-deleted are splice variants with the nine nucleotide deletion in exon 9.

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References

1. Martignetti, John A. et al. "Diaphyseal Medullary Stenosis with Malignant Fibrous Histiocytoma: a Hereditary Bone Dysplasia/ Cancer Syndrome Maps to 9p21-22." The American Journal of Human Genetics (1999) 64.3: 801-07.

2. Camacho-Vanegas, Olga et al. "Primate Genome Gain and Loss: A Bone Dysplasia, Muscular Dystrophy, and Bone Cancer Syndrome Resulting from Mutated Retroviral-Derived MTAP Transcripts" The American Journal of Human Genetics. (2012); 90(4):614-27.

3. Kim, Eddo et al."Insights into the Connection between Cancer and Alternative Splicing." Trends in Genetics (2008) 24.1: 7-10.