Alternative splicing of MEFV in mouse spleen cells

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Abstract:

Mutations in the MEFV gene are responsible for an auto inflammatory disorder known as Familial Mediterranean fever (FMF). FMF is characterized by recurrent episodes of fever and painful inflammation attacks in the chest, joints, and/or abdomen, which may last hours to a few days. The MEFV gene encodes a protein called pyrin, which has several roles involved in regulating innate immunity through the pathway that leads to the activation of interleukin-1β. Although FMF is inherited in a recessive manner, there are often cases of individuals heterozygous for a mutation in MEFV showing clinical symptoms of FMF. The purpose of this study is to analyze the impact of various compounds on MEFV expression, and to investigate any alternatively spliced transcript variants that are produced. The long-term goal is to find compounds that increase the amount of functional pyrin produced, which may be used to treat FMF in heterozygous individuals. Mouse spleen cells were treated with various compounds, and RNA samples isolated from the cells were used for RT-PCR analysis. Two transcript variants were found to be present in the cells, and sequencing analysis determined one transcript has an additional 90 bases spliced into exon 2 from intron 2. Two compounds were found to increase the relative prevalence of the +90bp variant. Future studies should be done on the protein level to better understand the isoforms' biological significance.

Introduction:

The MEFV gene is located on the short arm of chromosome 16. It contains 10 exons, and encodes a protein called pyrin. Pyrin is primarily expressed in certain white blood cells, such as monocytes, granulocytes, and dendritic cells, as well as in synovial fibroblasts. Five major domains are found in the protein: a pyrin domain, a bZIP transcription factor basic domain, a B-box zinc finger domain, an α-helical (coiled-coil) domain, and a B30.2 (PRYSPRY) domain. Pyrin is involved in regulating innate immunity, and has both pro- and anti-inflammatory roles, which impact the interleukin-1β activation pathway (Chae, 2009).

The pro-inflammatory role of pyrin is carried out via its N-terminal pyrin domain (PYD). When the cell recognizes some sort of foreign or danger signal, it prompts the formation of an inflammasome – a complex of proteins including pyrin, ASC (apoptosis-associated speck-like protein with a caspase-recruitment domain), and pro-caspase 1. Both pyrin and ASC contain PYDs, which bind to each other within the inflammasome. When these proteins are brought within proximity of each other it leads to the cleavage and activation of pro-caspase 1 into caspase 1. Caspase 1 then carries out the proteolytic activation of interleukin-1β, which triggers inflammation and fever (Manukyan, 2016).

The anti-inflammatory role of pyrin is carried out via its C-terminal B30.2 domain. This domain has binding sites for the active subunits of caspase 1. Binding the subunits inhibits them from causing the activation of interleukin-1β (Chae, 2009). Once activated, caspase 1 can also act on pyrin itself, cleaving it roughly in half. The N-terminal half of pyrin will then localize to the nucleus and exert influence on the NF-κB pathway (Chae, 2008). Moreover, pyrin has been shown to polarize to the leading edge of migrating monocytes and to co-localize to polymerizing

actin in previous studies, suggesting a role in migration of these cells to sites of inflammation (Waite, 2009).

Mutations in MEFV that affect pyrin function are responsible for an auto inflammatory disorder known as Familial Mediterranean fever (FMF). This disorder is one of the most frequent genetic disorders in Mediterranean populations, including Turkish, Arab, Armenian, and Jewish (Jéru, 2013). In these at-risk populations, FMF has a frequency ranging from 1 in 200 to 1 in 1,000. FMF is characterized by recurrent episodes of fever and painful inflammation attacks in the chest, joints, and/or abdomen, which may last hours to a few days (Jéru, 2013). The characteristics and severity of the disorder vary from patient to patient, namely due to the specific mutation they inherit (Touitou, 2001). This variation can be in how long the attack lasts, how frequent the patient has them, and which body part the inflammation is localized to. Between attacks, individuals with FMF are asymptomatic.

Although there are up to 80 disease causing mutations found in the MEFV gene, there are five mutations that account for 70-80% of FMF cases (Touitou, 2001). These mutations are: V726A, M694V, M694I, M680I and E148Q. While M694V and M680I have been shown to be associated with more severe forms of the disorder, E148Q has been found to be the least severe (Shinar, 2000). Like each of these founder mutations, the overwhelming majority of MEFV mutations are missense mutations.

Current treatment for FMF is daily and life-long colchicine administration (Jéru, 2013). Colchicine is characterized as a microtubule polymerization inhibitor. Though it is successful in preventing attacks, some patients are resistant or unable to tolerate its toxicity (Chae, 2009). Colchicine also prevents AA-amyloidosis, which is abnormal protein deposits and accumulation

in tissue due to prolonged inflammation. AA-amyloidosis is commonly seen in FMF patients, and can lead to terminal renal failure (Jéru, 2013).

FMF is inherited in an autosomal recessive manner; however, there are often cases of heterozygotes with only one mutated allele showing clinical symptoms of FMF. Heterozygous individuals actually account for up to 30% of FMF patients (Chae, 2009). The idea for this study stemmed from these cases, namely that environmental cues could be triggering episodes by reducing the amount of functional pyrin produced by the one wild-type allele. The purpose of this study is to analyze the impact of various compounds on MEFV expression, and to investigate any alternatively spliced transcript variants produced. The long-term goal is to find compounds that increase the amount of functional pyrin produced, which could potentially be used to treat FMF in heterozygous individuals.

Materials and Methods:

Cells were harvested from mice spleens, which were removed from sacrificed mice. The cells were then treated with various agents for 24 hours. Untreated cells were used as a control.

RNA Purification:

After 24 hours, RNA was isolated from the cells. First, cells were transferred to 1.5mL Eppendorf tubes, and centrifuged to form a pellet. Then, media was removed off the top, and the pellet was resuspended in RLT lysis buffer plus B-mercaptoethanol. A QIAGEN QIAshredder spin column was used to remove genomic DNA. RNA was purified using the QIAGEN RNeasy Plus Mini Kit, while following the protocol in the QIAGEN RNeasy Plus Mini Handbook (2010).

RNA samples were quantified using a spectrophotometer (Beckman DU-500), and the readings were used to make 5ng/µl stocks for the RT-PCR reactions.

RT-PCR:

RT-PCR analysis was performed on the RNA samples utilizing the QIAGEN OneStep RT-PCR Kit, following the protocol in the Handbook (2010). Two different primer pairs were used: the first spanned exon 1 to exon 8, while the second spanned exon 2 to exon 3. The primer sequences are as follows: exon 1 forward – GAGGAGTATGCTGTAAGGCTGAC, exon 8 reverse – GAGAATGGATCTCATGTAGCTC; exon 2 forward – CAGCATACTCTCTGATCCAG, exon 3 reverse – CACTCGATGTCCTTGATGCTC.

The thermal cycler for the amplification from exon 1 to exon 8 was set for 40 cycles with 10µl reactions. The first hold was set at 50°C for 30min, then 94°C for 15min. Then, the cycles were programmed as follows: 94°C for 30sec, 58°C for 30sec, and 72°C for 2min 15sec. A final extension time was set for 72°C for 7min, then the thermal cycler will store the reaction at 4°C.

The thermal cycler for the amplification from exon 2 to exon 3 was set for 40 cycles with 10µl reactions. The first hold was set at 50°C for 30min, then 94°C for 15min. Then, the cycles were programmed as follows: 94°C for 30sec, 58°C for 30sec, and 72°C for 35sec. A final extension time was set for 72°C for 7min, then the thermal cycler will store the reaction at 4°C.

Gel Electrophoresis:

PCR products were visualized by carrying out gel electrophoresis on a 15cm 1% agarose gel (200ml of 1xTBE and 2g of agarose) with ethidium bromide (16.5ml of 10mg/ml stock).

Sequencing:

PCR products were purified following QIAquick RNA Purification Spin Protocol (2008), and sent to GeneWiz for sequencing.

ImageJ Quantification:

Band intensities were quantified utilizing ImageJ software, and a ratio of upper band to lower band intensities was calculated for each sample.

Results:

The first amplification used a primer pair spanning exon 1 to exon 8 (Figure 1). The results show that two MEFV transcript variants are being expressed in the cells. Sequencing analysis of the purified PCR products determined that the two variants differ by 90 bases. The shorter transcript matched the expected sequence for MEFV, while the longer transcript contained the same sequence, but also included the first 90bp of intron 2 (Figure 2).

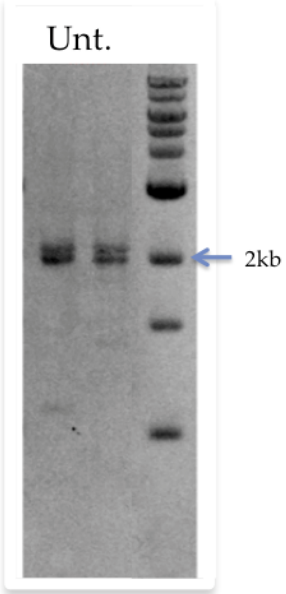

Figure 1. RT-PCR results show two transcript variants are expressed. Primer pair used spans exon 1 to exon 8, and products were visualized on a 1% agarose gel with EtBr.

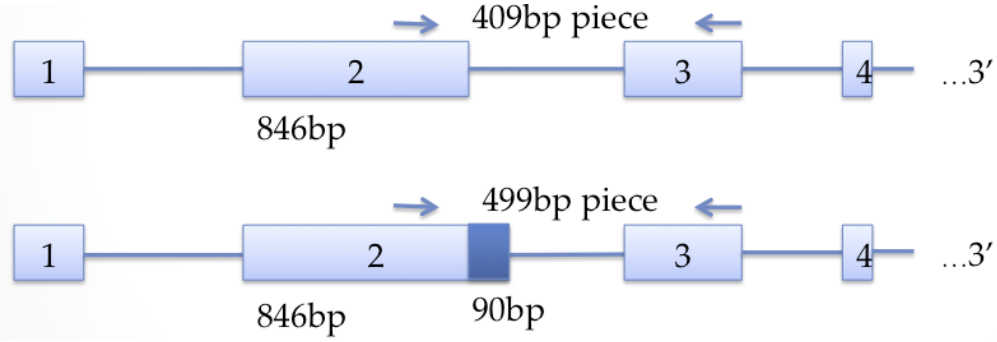

Figure 2. The two MEFV transcript variants based on sequencing analysis. Arrows represent primer pair used in subsequent RT-PCR reaction.

The amplification in Figure 3 used a primer pair spanning exon 2 to exon 3 (primer pair shown in Figure 2). The two transcript variants were present in all of the cells. In Figure 3, numbers at the top of the lanes correspond to compounds used to treat the cells, while numbers at the bottom of the lanes correspond to ratios of upper to lower band intensities.

ImageJ software was used to quantify the intensities of the upper and lower bands of each sample. Then, a ratio of the upper band (+90bp transcript) divided by the lower band was calculated. While most of the samples had a ratio within the range of 1.18 – 1.38, compound 32 had a ratio of 1.87 and compound 205 had a ratio of 2.71 (Figure 3).

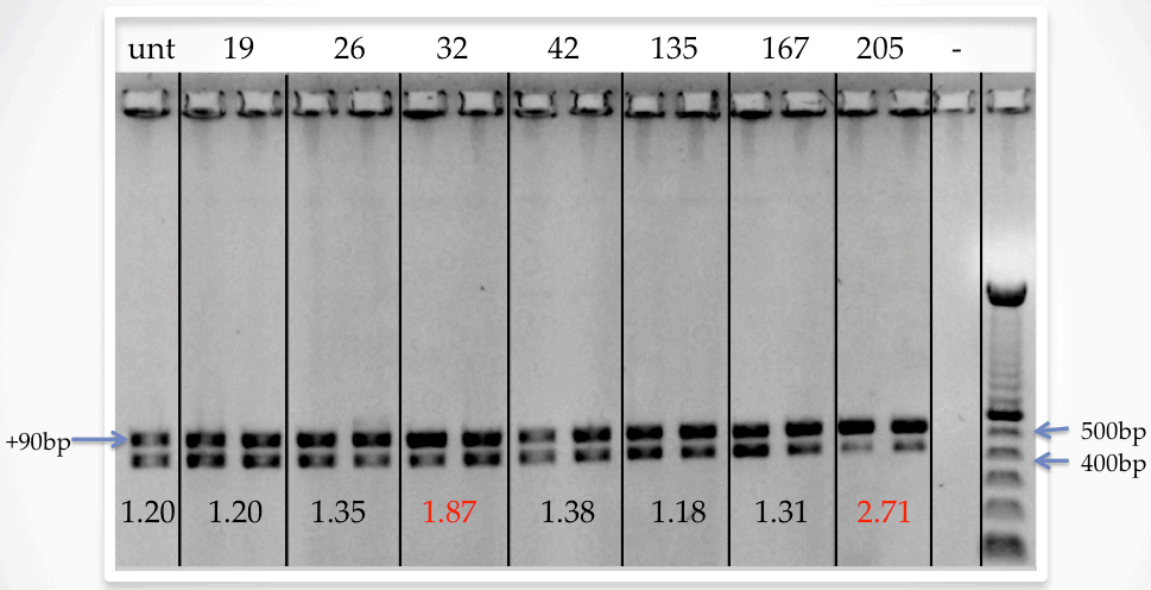

Figure 3. RT-PCR results and band intensity ratios. Primer pair spanned exon 2 to exon 3, and products were visualized on a 1% agarose gel with EtBr.

Discussion:

In this study, it was found that two MEFV transcript variants are produced in mouse spleen cells. Upon sequencing analysis, it was determined that these transcripts differ by 90 bases in exon 2. The longer transcript has an extra 90 bases, which are spliced into the transcript from intron 2. This +90bp transcript is likely produced due to a sequence within intron 2 (GTTAGT), which is nearly identical to the donor splice site consensus sequence (GTRAGT). Exon 2 codes for a region in the protein's N-terminus, which has been shown to function in microtubule binding, as well as localization to the nucleus when pyrin is cleaved by caspase 1. The +90bp transcript remains in frame, and will introduce an additional 30 amino acids into this region of the protein. If this inclusion has an effect on protein function, it may have implications in FMF.

The results suggest that treatment with certain agents can manipulate alternative splicing of these MEFV transcripts. The bands corresponding to the RT-PCR products from untreated

cells in Figure 3 appear to be approximately equal in intensity, suggesting that there are relatively equal amounts of the two transcript variants present in these cells. To determine whether or not the various agents used to treat the other cell samples impacted the relative prevalence of these transcripts, ImageJ software was used to quantify a ratio of the upper band (+90bp transcript) intensity divided by the lower band intensity. While most of the samples' ratios range from 1.18 – 1.38, compound 32 reached 1.87 and compound 205 reached 2.71 (Figure 3). This demonstrates an increase in the relative prevalence of the +90bp transcript in these cells compared to the shorter transcript.

Further studies should be done on the protein level to better understand the biological significance of the two MEFV isoforms, preferably utilizing an appropriate immortalized human cell line.

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