IKBKAP mRNA in Peripheral Blood Leukocytes: A Molecular Marker of Gene Expression and Splicing in Familial Dysautonomia

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ABSTRACT: The common familial dysautonomia (FD) mutation results in tissue specific mis-splicing with reduced amount of wild-type (WT) I κ B kinase associated protein gene (*IKBKAP*) mRNA and ELP1. ELP1 is a subunit of Elongator, formerly called the I κ B kinase associated protein (IKAP) protein. We measured *IKBKAP* mRNA in peripheral blood leukocytes to determine whether FD subjects and carriers have characteristic levels. Estimated mean *IKBKAP* mRNA levels, measured by quantitative PCR and expressed as amount relative to the noncarrier average, were significantly different for the two groups when not adjusted for age and sex ($p < 0.001$): FD subjects 0.23, 95% confidence interval (CI) (0.19, 0.28); carriers 0.58, 95% CI (0.50, 0.68); or adjusted for age and sex ($p < 0.001$): FD subjects 0.21, 95% CI (0.16, 0.26); carriers 0.66, 95% CI (0.55, 0.79). Comparison of *IKBKAP* mRNA levels of the 22 FD subjects and their related carriers showed a strong correlation, providing evidence for genetic control of splicing efficiency. *IKBKAP* mRNA levels were not higher in those subjects using tocotrienols or epigallocatechin gallate. Levels of *IKBKAP* mRNA in peripheral blood leukocytes can be used to assess molecular response to therapies aimed at enhancing exon 20 inclusion and increasing cellular levels of ELP1/IKAP. (*Pediatr Res* 63: 186–190, 2008)

Familial dysautonomia (FD), also known as Riley Day syndrome or hereditary sensory and autonomic neuropathy type III (HSAN III), is characterized by pervasive and variable sensory and autonomic dysfunction (1,2). FD primarily affects the development of unmyelinated and small myelinated neurons and is associated with progressive neuronal degeneration. Multiple systems are impacted, resulting in a markedly reduced quality of life and premature death (1–3).

In 2001, two mutations that cause FD in the Ashkenazi Jewish population were identified in the I κ B kinase associated protein gene, *IKBKAP* (4,5). The most common FD mutation is a single T to C DNA change located at the sixth base pair of intron 20 (4,5). This mutation causes a splicing defect that leads to variable skipping of exon 20 in *IKBKAP* mRNA and reduces the cellular level of I κ B kinase associated protein (IKAP). IKAP was originally identified as an I κ B kinase

(IKK) complex-associated protein. However, this name is now a misnomer as subsequent work has disproved the role of IKAP in this pathway. IKAP, or ELP 1, is a subunit of the highly conserved complex Elongator, which is involved in transcriptional elongation (6). All FD individuals tested to date carry at least one copy of the IVS20 + 6T > C mutation, with more than 99.5% being homozygous for the mutation, and the remainder being heterozygous for R696P (4,5). To date, only one other FD mutation has been described, P914L, in a patient of mixed ancestry (7).

The IVS20 + 6T > C mutation does not lead to a complete loss of function. Instead, it results in a tissue-specific decrease in splicing efficiency of the *IKBKAP* transcript. Importantly, FD patients retain the capacity to produce some wild-type (WT) mRNA and IKAP or ELP1 protein (5,8). Evaluation of *IKBKAP* mRNA levels in FD patient tissues shows that the most profound mis-splicing occurs in neuronal tissue and leads to very low levels of functional *IKBKAP*. The amount of WT *IKBKAP* varies in somatic tissues, with lymphoblast cell lines established from FD patients expressing the highest level of WT *IKBKAP* (8).

Recently, various compounds that modify *IKBKAP* splicing or expression have been identified and have been suggested as possible therapeutic agents for FD (9–12). Two compounds are nutraceuticals: tocotrienols (an unsaturated form of vitamin E) and epigallocatechin gallate (EGCG, a component of green tea) (9,10), whereas the third is a plant cytokinin, kinetin (11,12). To date, reports regarding usefulness of these compounds have been limited to cell culture observations. Therefore, objective measures of drug efficacy must be established before initiating clinical studies in FD patients.

The fact that neuronal tissue shows the greatest reduction of WT *IKBKAP* is consistent with the pervasive neurologic abnormalities seen in FD. However, since other tissues also demonstrate a decrease in *IKBKAP* levels, we hypothesized that peripheral blood leukocytes would show a characteristic decrease in *IKBKAP* mRNA expression in FD individuals, as well as in carriers of the major mutation. The ability to

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Abbreviations: EGCG, epigallocatechin gallate; FD, familial dysautonomia; IKAP, I κ B kinase associated protein; *IKBKAP*, I κ B kinase associated protein gene

measure *IKBKAP* levels in peripheral blood, an easily obtainable clinical sample, will provide a molecular marker of gene expression and splicing that can be used to monitor patient response to potential therapeutic agents aimed at increasing splicing efficiency in this progressive and fatal disease.

METHODS

Study population. At total of 95 subjects (FD subjects, FD carriers, and noncarriers for calibration of assay) were enrolled in the study. FD subjects were recruited from the FD registry database at the Dysautonomia Treatment and Evaluation Center at the New York University Medical Center. All 45 FD subjects fulfilled clinical diagnostic criteria for FD and were known to be homozygous for the common mis-splicing mutation. We did not discontinue any routine medications in our FD subjects, but noted that 30 of the 45 FD subjects were taking fludrocortisone, a medication that has been reported to alter splicing (13). In addition, 8 of the 45 FD subjects were taking nutraceuticals that might also affect total *IKBKAP* mRNA production; six subjects were taking tocotrienols and two subjects were taking both tocotrienols and EGCG for more than three years (9,10). None of the individuals taking nutraceuticals were also taking fludrocortisone.

The carrier group was comprised of 26 individuals who were either parents or siblings of FD subjects. The noncarrier group was comprised of 24 individuals who were noncarrier siblings from the FD families, as well as healthy volunteers from non-FD families. Both carriers and noncarriers had their genetic status verified by independent molecular DNA analysis before this study and volunteered this genetic information. For the 45 FD subjects, of whom 2 were siblings, there were 22 related carriers enrolled allowing us to analyze the influence of family membership.

All participants were of Ashkenazi Jewish descent, with the exception of one noncarrier who was Asian. The NYU School of Medicine institutional review board approved this study and informed consent was obtained from all participants.

Blood collection and RNA analysis. Each participant had a single sample of blood taken for RNA extraction using the PAXgene Blood RNA Kit (PreAnalytiX-Qiagen/BD, Germantown, MD). Each blood sample was identified by a coded number and sent to the Center for Human Genetic Research at Massachusetts General Hospital for RNA extraction and quantification using UV absorbance. Reverse transcription was performed using 500 ng total RNA, oligo(dT)/random hexamer primers (Promega, Madison, WI), and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). q-PCR (quantitative-PCR) analysis of WT human *IKBKAP* was performed using the Bio-Rad iCycler, iQ SYBER Green supermix (Bio-Rad, Hercules, CA) and primers, F: 5'-TTCACGGATTGCTACTGTTGTG-3' and R: 5'-TGTC-CAACCACTTCCGAATCTGAG-3', specific to the WT spliced isoform of *IKBKAP*.

To ensure that the same amount of starting cDNA was present in each tube, samples were normalized to the human *GAPD* gene, a reference gene with consistent expression in mRNA isolated from peripheral blood samples. Two *GAPD* primers were used, F: 5'-TGCACCACCAACTGCTTAGC-3' and R: 5'-GGCATGGACTGTGGTCATGAG-3'. The primer pairs were tested against a standard curve and the correlation coefficient (r^2) and amplification efficiencies all fell within an accepted range. q-PCR was performed in triplicate on each sample and the relative amount of WT *IKBKAP* was measured between the samples using the Livak method for relative gene expression analysis (14). This method measures the difference in each sample (ΔCt) between the threshold cycles of the target gene (*IKBKAP*) to the reference gene (*GAPD*). The mean ΔCt for each triplicate was graphed and SD for each mean were calculated. We used the average ΔCt of the noncarriers as the calibrator, therefore the reported *IKBKAP* mRNA levels for each FD and carrier sample are expressed relative to the average noncarrier *IKBKAP* mRNA level.

Statistical methods. Demographic characteristics of FD subjects and carriers were compared using an independent samples *t* test (age) and a χ^2 test (sex). Independent sample *t* tests were used to ascertain whether FD subjects who were taking tocotrienols with or without EGCG had different *IKBKAP* mRNA levels than the FD subjects not taking these supplements and whether FD subjects taking fludrocortisone had different *IKBKAP* mRNA levels than the FD subjects not taking fludrocortisone.

Since the distribution of *IKBKAP* mRNA levels was positively skewed, a square root transformation was used to transform the data for statistical analyses that require the assumption of normality of data. After statistical analysis, *IKBKAP* levels were back-transformed to present results in their original units for description.

Mixed model analyses of covariance and variance were used to compare *IKBKAP* levels among the FD subjects and carriers with and without adjustment for age and sex. Mixed models take into account the correlations between observations within the same primary unit, that is, family membership. This analysis allowed us to include multiple subjects from the same group (*i.e.* FD or carrier) within a particular family.

Mixed model analyses of variance were also used to study the association between sex and *IKBKAP* mRNA level separately for FD subjects and carriers. Spearman rank correlation coefficients were used to assess the association between age and *IKBKAP* mRNA level separately for the FD subjects and the carriers.

Box plots were used to graphically display the distribution of *IKBKAP* mRNA levels for various groups being compared. To demonstrate the influence of family membership, a multiple line graph was used to display the relative *IKBKAP* mRNA expression levels for the 22 FD subjects and their respective related carriers. A forward stepwise linear regression procedure was used to assess the effect of age and sex on the association between carrier and FD subject *IKBKAP* mRNA levels within families for the 22 families with FD subjects and carriers. Carrier *IKBKAP* mRNA level was used as a predictor of FD *IKBKAP* mRNA level.

Analyses were performed using SAS v9.1.3. (SAS Institute, Cary, NC) box plots and a multiple line graph were produced using SPSS v14.0 (SPSS Inc., Chicago, IL).

RESULTS

Group demographic characteristics (FD subjects and carriers). The 45 FD subjects (mean age 21.1 ± 12.9 y) were significantly younger than the 26 carriers (mean age 46.1 ± 12.8 y, $p < 0.001$). There were a higher proportion of males in the FD group (55.6%) than in the carrier group (30.8%, $p = 0.04$). Since the mean ages and the sex distributions of FD subjects and the carriers differed, we conducted the analyses of the association of *IKBKAP* mRNA levels and subject type with and without the use of age and sex as covariates. Thirty of the 45 subjects were taking fludrocortisone. The difference in mean *IKBKAP* mRNA levels between subjects taking fludrocortisone [0.26, 95% CI (0.20, 0.33)] and the 15 subjects not taking fludrocortisone [0.24, 95% CI (0.17, 0.30)] was small.

For the eight FD subjects in this analysis who were taking either tocotrienols and/or EGCG, the mean *IKBKAP* mRNA level was 0.21, 95% CI (0.10, 0.36); the mean *IKBKAP* mRNA level for the 37 FD subjects who were not taking these supplements was similar 0.24, 95% CI (0.20, 0.28). Subjects taking the supplements and subjects taking fludrocortisone were included in the subsequent analyses.

Association of *IKBKAP* mRNA and subject type (FD subject and carrier). Figure 1 displays a set of box plots showing the distributions of *IKBKAP* mRNA for the FD subjects and carriers. The distributions of *IKBKAP* mRNA levels in the two groups are distinctly different, with little overlap of values common to both groups.

Table 1 presents results derived from the mixed model ANOVA and the mixed model analysis of covariance (ANCOVA) that compared *IKBKAP* mRNA levels between the FD subjects and the carriers. The table displays two sets of least square means for the *IKBKAP* mRNA levels with 95% CI. In both of these analyses, subject type (FD or carrier) was significantly associated with *IKBKAP* mRNA ($p < 0.001$) with and without adjustment for age and sex. Similarly, in the ANCOVA analysis, where age and sex were used as covariates, subject type also was significantly associated with *IKBKAP* mRNA ($p < 0.001$). The least squares estimates resulting from the multivariate ANCOVA analysis are relatively close in value

Figure 1. Levels of *IKBKAP* mRNA in FD subjects (45) and carriers (26) as determined by q-PCR.

to those produced by the univariate ANOVA analysis, underlining the weak effect of age and sex on the association between group membership and *IKBKAP* mRNA levels.

Within each group of FD subjects and carriers, analyses of associations between *IKBKAP* mRNA levels and both age and sex showed that for each group, *IKBKAP* mRNA levels were weakly associated with both age and sex. *IKBKAP* mRNA levels diminished slowly with age with very weak correlations between age and *IKBKAP* mRNA levels in both the FD subject group (Spearman's $r = -0.23$, $p = 0.14$ for FD subjects) and the carriers (Spearman's $r = -0.22$, $p = 0.29$ for carriers). Sex was not associated with *IKBKAP* mRNA level for either the FD subjects (0.56) or the carriers ($p = 0.58$). For the FD subjects, estimates of mean *IKBKAP* mRNA levels with 95% CI were 0.25 (0.006–0.86) for males and 0.22 (0.006–0.073) for females. For the carriers, estimates of mean *IKBKAP* mRNA levels were 0.63 (0.44–0.85) for the males and 0.57 (0.35–0.86) for the females.

Association of *IKBKAP* mRNA levels in related individuals. Examination of Figure 2 shows a striking relationship between *IKBKAP* mRNA levels in the 22 related FD/carrier pairs. In those families with more than one FD subject or carrier, *IKBKAP* mRNA levels were averaged for the FD subjects and carriers.

For one related FD-carrier pair, the FD subject's *IKBKAP* mRNA level exceeded the carrier's level and was 0.07 units higher. However, only one obligate carrier from that family membership was represented. For all other families, *IKBKAP* mRNA levels were higher for the carriers than for the FD subjects as expected. For four (18%) of the families, the

Figure 2. Relationship between *IKBKAP* mRNA level of FD subjects and related carriers in 22 FD families. FD subject mRNA levels are shown on the left, carrier mRNA levels are on the right. Lines were drawn between levels of related pairs.

carriers' *IKBKAP* mRNA levels were less than 20 above the levels for the FD subjects. For eight (36%) of the families, this difference was between 0.20 and 0.39; for five (23%) of these families, the differences were between 0.40 and 0.50; for three (14%) of these families, the differences were between 0.72 and 0.90. Comparing the relative levels of the FD subjects and the carriers, for half the families, the *IKBKAP* mRNA levels for the carriers were up to 2.5 times the levels for the FD subjects; for three families, the *IKBKAP* mRNA levels for the carriers were greater than 4 times the levels for the FD subjects, reaching as high as 7.6 for one family.

DISCUSSION

Familial dysautonomia is a hereditary sensory and autonomic neuropathy with recessive inheritance that affects neuronal development and survival. FD is caused by mutations in the *IKBKAP* gene that lead to a tissue specific reduction in IKAP or ELP1 levels. The fact that every affected FD individual has at least one copy of the mis-splicing mutation suggests that the observed tissue specific variability is required for development of the disease.

ELP1 is a member of the human Elongator complex (6). Elongator plays a role in transcriptional elongation and it has

Table 1. Least squares estimates of *IKBKAP* mRNA levels in FD subjects and carriers expressed as a percentage relative to *IKBKAP* levels in noncarrier subjects

Group	No.	Mean	No adjustment for age or sex (ANOVA) 95% Confidence limits		Mean	Adjusted for age and sex (ANCOVA) 95% Confidence limits	
			Lower	Upper		Lower	Upper
FD subjects	45	0.23	0.19	0.28	0.21	0.16	0.26
Carriers	26	0.58	0.50	0.68	0.66	0.55	0.79

been demonstrated that reduction of ELP1, as in FD, results in the down-regulation of a large number of target genes (6,15). Many of Elongator's target genes are involved in cell proliferation and migration, which may explain the observed FD neuropathology that is consistent with arrested small fiber neuronal development and progressive neuronal loss resulting in variable degrees of sensory and autonomic dysfunction (1,2).

To date, treatment for this fatal disease is supportive and preventative. However, given the frequency of the major mutation there have been efforts to find agents that will modify mRNA splicing and alter *IKBKAP* expression (9–12). As we postulate and justify therapeutic interventions for affected individuals with such agents that affect the splicing mechanism *in vitro*, we appreciate the need to identify biomarkers of disease expression, which will enable us to monitor treatment efficacy and possibly neurologic progression and disease outcomes. Thus, our primary aim was to determine whether the effect of the splicing mutation could be detected in readily accessible cells such as peripheral blood leukocytes.

As expected, our data indicate that there are distinct ranges of *IKBKAP* mRNA levels in peripheral blood leukocytes in FD subjects and carriers. In general, in peripheral blood leukocytes, carriers had a mean *IKBKAP* mRNA level that was 61% of noncarrier levels, which is a more profound decrease than in that observed in cell lines (8). We also demonstrated that differences in *IKBKAP* mRNA levels between FD subjects and related carriers in 22 related FD-carrier pairs were remarkably similar suggesting strong familial relationships in gene expression and splicing regulation.

Due to the demographic composition of the population available for study, there were noticeable differences between mean ages and gender among the FD subjects and the carriers. The majority of FD participants were accompanied to our Center by their mothers; this accounts for the higher percentage of females in the carrier group. However, since the mixed model analysis of variance and covariance showed that *IKBKAP* mRNA levels were significantly related to study cohort, and the least square estimates were similar in both analyses, we conclude that our results are not biased by the imbalances among our subject groups and a strong association between group membership and *IKBKAP* mRNA levels remains. Thus, group membership (carrier *versus* FD) is a much stronger determinant of *IKBKAP* mRNA level than age and sex.

Although several agents have been shown to alter splicing at the *IKBKAP* locus *in vitro*, no studies to date have been performed with these agents *in vivo*. In studies using FD cell lines, fludrocortisone, a commonly used medication in the FD population, has been shown to decrease expression of WT *IKBKAP* mRNA, and two nutraceuticals that are available over-the-counter (tocotrienols and EGCG) have been shown to increase expression of WT *IKBKAP* mRNA (9,10,13). Thirty of the 45 subjects used fludrocortisone daily. Eight subjects used tocotrienols daily and two of these subjects also used EGCG daily; none of these 8 subjects were also taking fludrocortisone. Neither fludrocortisone nor use of nutraceuticals significantly affected our data, which allowed all FD subjects to be considered as a single group. We acknowledge

that the sample size for the cohort taking nutraceuticals was small ($n = 8$), and there was not sufficient power to detect a difference in the range of the actual data. However, for the purpose of understanding whether the supplements affected results, we were primarily interested in the magnitude, not the significance of the difference. As was expected, due to the sample size, there was no significant difference between *IKBKAP* mRNA levels between subjects taking and not taking fludrocortisone. However, it is possible that the *in vivo* splicing effect of certain agents differ from *in vitro* findings due to an inability to achieve similar tissue concentrations *in vivo* or due to variability in *IKBKAP* mRNA levels between FD individuals.

What is particularly striking is that there appears to be some genetic control over splicing. Our data show that those FD subjects with low levels of *IKBKAP* mRNA are more likely to be related to the individual carriers who also have lower levels of *IKBKAP* mRNA; and those FD subjects with higher levels are similarly more likely to be related to the individuals with relatively higher levels of *IKBKAP* mRNA. When the mRNA expression levels are ranked lowest to highest in the FD and in the carrier groups, a strong correlation is noted between related pairs. In fact, many of the lines representing the differences between *IKBKAP* levels for the FD subjects and carriers within a family are relatively parallel as can be seen in Figure 2, indicating that the magnitude of the difference in *IKBKAP* levels between FD subjects and carriers in the same family is similar for the majority of families. Although only one related carrier per FD subject was included, these results suggest that other genetic factors influence splicing fidelity.

Our analysis confirms that ineffective splicing in both FD subjects as well as carriers of the FD mutation can be detected in peripheral blood leukocytes. Furthermore, the predictability and consistency of *IKBKAP* levels in FD subjects as well as the ease of obtaining repeated blood samples in a relatively noninvasive manner validates the use of *IKBKAP* in peripheral blood leukocytes as a biomarker of gene expression and splicing efficiency. Nonetheless, we acknowledge that *IKBKAP* splicing and expression is tissue-specific, and *IKBKAP* mRNA levels in peripheral blood samples may not reflect absolute levels in neuronal tissue.

In conclusion, our data indicate that sampling RNA from peripheral blood leukocytes is a reliable and noninvasive method to measure *IKBKAP* in FD individuals. This assay provides one method of following genetic expression and splicing efficiency in FD, which can be important for the development of new therapies that may modify this devastating disease.

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