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EGCG corrects aberrant splicing of IKAP mRNA in cells from patients with familial dysautonomia

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Abstract

Familial dysautonomia (FD) is an autosomal recessive neurodegenerative disorder. The most prevalent causative mutation is a T → C transition in a donor splice site of the *IKBKAP* transcript, resulting in aberrant splicing and a truncated protein. The mutation's position and leaky nature suggested that its impact might be moderated by altering the level of splice-regulating proteins. The reported ability of (−)-epigallocatechin gallate (EGCG), a polyphenol, to down-regulate the expression of hnRNP A2/B1, a *trans*-activating factor that encourages the use of intron-distal 5′ splice sites, prompted an evaluation of its effect on the *IKBKAP* transcript in FD-derived cells. EGCG reduces the level of hnRNP A2/B1 and increases the amounts of the wild-type *IKBKAP*-encoded transcript and functional protein. Combined treatment of cells with EGCG and tocotrienol, which upregulates *IKBKAP* transcription, results in a synergistic production of the functional gene product. These findings suggest the possible use of EGCG as a therapeutic modality for individuals with FD.
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Keywords: Familial dysautonomia; (−)-Epigallocatechin gallate; IKAP; *IKBKAP*; Alternative splicing; hnRNP

Regulated alternative splicing of pre-mRNA is a critical mechanism by which functionally different proteins are generated from the same gene. Pre-mRNA splicing is carried out by spliceosomes which are multicomponent ribonucleoprotein complexes containing small nuclear RNAs and a large number of associated proteins. Splice site selection and specificity are influenced by 5′ and 3′ splice sites located at the exon–intron boundaries of pre-mRNAs and by exonic splicing enhancer (ESE) and suppressor (ESS) elements [1–4]. In general, the binding of serine/arginine rich proteins (SR proteins) to the ESEs enhances splicing and the binding to the ESSs by members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family results in a suppression of splicing. In vitro and in vivo studies reveal that SR proteins stimulate the selection of intron-proximal 5′ splice sites in pre-mRNAs that contain two or more alternative 5′ splice sites, while hnRNPs have the opposite effect, promoting the selection of intron-distal 5′ splice sites [5–7]. The extensively studied hnRNPs of the A/B group exhibit significant amino acid sequence homology and changes in cellular amounts or activities of these proteins mediate alternative patterns of RNA processing of cellular and viral transcripts [6–11].

Familial dysautonomia (FD) is an autosomal recessive disorder primarily confined to individuals of Ashkenazi Jewish descent that affects the development and survival of sensory, sympathetic, and some parasympathetic neurons [12–14]. FD is caused by mutations in the gene termed *IKBKAP* which encodes a protein termed IkB kinase complex-associated protein (IKAP) [15,16]. IKAP, which was originally reported to be a scaffold protein involved in the assembly of the IkB kinase complex [17], is more likely a component of the Elongator complex [18,19] and/or is a c-Jun N-terminal kinase (JNK)-associated protein [20]. FD is caused by one of two known mutations. The more common, or major mutation, is a T → C transition in the sixth base of the donor splice site of intron 20, termed IVS20$^{+6T\rightarrow C}$, that changes the intron 20 donor splice site sequence from the consensus GTAAGT to a non-consensus GTAAGC

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and results in the production of an IKAP transcript lacking exon 20. The less common or minor mutation is a G→C transversion that results in an arginine to proline substitution of amino acid residue 696 of IKAP.

The observed ability of tissues and cells derived from individuals with FD to produce some exon 20-containing, or wild-type, transcripts [21,22] suggested that the FD phenotype might be modulated through the production of variable amounts of the functional gene product. Anderson et al. (2003) recently demonstrated that tocotrienols, members of the vitamin E family, can upregulate transcription of the *IKBKAP* gene. This increased expression results in an increased production of both the truncated and full-length transcripts and an increase in the amount of functional IKAP [21]. FD-affected individuals receiving tocotrienol supplementation have had some amelioration of their symptoms (unpublished observations).

In FD patients, the causative mutation results in the preferential use of an intron-distal 5′ splice site, the consequence of which is the exclusion of exon 20 and the generation of a truncated IKAP. Noting the ability of (−)-epigallocatechin gallate (EGCG) to down-regulate hnRNP A2/B1 protein and gene expression [23] and the ability of the hnRNPs to promote selection of intron-distal 5′ splice sites [5–7], we investigated whether EGCG treatment of FD-derived cells would increase the use of the intron-proximal splice site, in turn resulting in the generation of more exon 20-containing IKAP transcript. We report a reduction in the level of hnRNP A2/B1 mRNA and proteins and an increase in the amount of the exon 20-containing IKAP transcript in EGCG-treated FD-derived cells. We further report the synergistic production of correctly spliced transcript and full-length protein in cells treated with the combination of tocotrienol and EGCG.

Materials and methods

Cells and reagents. The GM00850 and GM04663 cell lines, homozygous for the IVS20$^{+6T\rightarrow C}$ FD-causing mutation, and the GM02912 cell line, derived from an unaffected individual, were obtained from the NIGMS Human Genetic Mutant Cell Repository. LA1-55n cells were kindly provided by Dr. Robert A. Ross. EGCG and δ-tocotrienol were purchased from Calbiochem. A monoclonal antibody generated against a peptide encoded by exons 23–28 of *IKBKAP* was purchased from BD Biosciences. The 4B10 and DP3B3 monoclonal antibodies, which recognize hnRNP A1 and hnRNP A2/B1, respectively, were kindly provided by Dr. Gideon Dreyfuss.

RNA preparation and real-time RT-PCR. Cells seeded in 96-well plates were treated in triplicate for approximately 24 h unless noted otherwise. After treatment, the cells were washed once with PBS and then lysed in 50 μl Cell-to-cDNA lysis buffer (Ambion) at 75 °C for 12 min. After one freeze–thaw cycle, 4 μl of lysate was used in 20 μl RT-PCR. The Quantitect SYBR Green RT-PCR Kit (Qiagen) was used for real-time RT-PCR analysis of the relative quantities of the exon 20-containing transcript (wild-type), the exon 20-lacking transcript (mutant), and exon 34–35-containing transcript that is unaffected by the FD-causing mutation (total), as well as the hnRNP A2/B1 and hnRNP A1 transcripts. The primers used for these analyses were as follows: for the exon 20-containing transcript, 5′-AGTTGTTCAT CATCGAGC-3′ and 5′-CATTTCCAAGAAACACCTTAGGG-3′; for exon 20-lacking transcript, 5′-CAGGACACAAAGCTTGTAT TACAGACTT-3′ and 5′-CATTTCCAAGAAACACCTTAGGG-3′; for exon 34–35-containing transcript, 5′-GAGATCATCCAAGAA TCGC-3′ and 5′-GGTAGCTGAATTCTGCTG-3′; for hnRNP A2/B1 transcript, 5′-GAGTTGTTTCTCGAGCAG-3′ and 5′-TGATCTT TTGCTTGCAGG-3′; and for hnRNP A1 transcript, 5′-TCGTGGA GGAAACTTCAGTG-3′ and 5′-TGTAGCTTCCACCACCTCCA-3′. Primers were used at a concentration of 0.5 μM. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems), programmed as follows, was used to perform the real-time RT-PCR and analysis: 50 °C × 30 min and 95 °C × 15 min for one cycle, followed by 40 cycles of 94 °C × 15 s, 57–60 °C × 30 s, and 72 °C × 30 s. To present relative amounts of PCR product obtained, results are expressed as changes in the threshold cycle (ΔC_T) compared to untreated cells. The threshold cycle refers to the PCR cycle at which the fluorescence of the PCR is increased to a calculated level above background. A change of 1.0 in C_T, assuming 100% PCR efficiency, would reflect a twofold change in the starting amount of the RNA template that was amplified.

To control for the amount of RNA present in the samples, RT-PCR amplification of ribosomal 18S RNA was performed on all cell lysates. The use of the ribosomal 18S RNA has been shown to be an effective control for the quantity of RNA present in samples [24]. For this analysis, TaqMan Ribosomal RNA Control Reagents were used with the TaqMan EZ RT-PCR Kit (Applied Biosystems) as per the manufacturer's protocol.

RT-PCR. Four microliters of cell lysates produced as described above was used in 20 μl RT-PCR using the One Step RT-PCR Kit (Qiagen) with the following primers which recognize sequences in exons 19 and 23 of *IKBKAP*: 5′-GCAGCAATCATGTGTCCCA-3′ and 5′-TAGCATCGCAGACAAGGTC-3′. The RT-PCR was carried out as follows: one cycle of 50 °C × 30 min and 95 °C × 15 min, followed by 41 cycles of 94 °C × 20 s, 60 °C × 30 s, and 72 °C × 15 s, and then a final extension of 72 °C × 2 min. PCR products were analyzed on a 2% agarose gel.

Western blot analysis. Cells treated as described in the figure legends were washed twice with PBS and lysed in 0.5 M Tris–HCl, pH 6.8, containing 1.4% SDS. Western blot analysis was performed essentially as described [25]. Equal amounts of protein fractionated on a 7% NuPAGE Tris–acetate Gel (Invitrogen) were blotted onto nitrocellulose (Bio-Rad) and probed overnight with antibody. The blots were then washed and probed with a goat anti-mouse antibody conjugated to alkaline phosphatase (Promega), followed by detection with Western blue substrate solution (Promega). All blots were also probed with an anti-actin antibody (Oncogene) to confirm equal protein loading.

Results

The almost complete absence of exon 20-containing *IKBKAP* transcript in cells bearing the IVS20$^{+6T\rightarrow C}$ mutation is somewhat surprising, as the presence of a C in the sixth position of a donor splice site occurs in approximately 15% of all donor splice sites [26]. The impact of the IVS20$^{+6T\rightarrow C}$ mutation on splicing prompted an examination of the exon 20 nucleotide sequence and revealed the presence of two putative ESS motifs (Fig. 1). Since alternative splicing is modulated by competition between, and the relative abundance of, factors that bind ESS and ESE elements, as well as the strength of the splice sites, we examined the effect of

ATGCCAAGGG GAAAC**TTAG**A AGTTGTTCAT
CATCGAGCCC TGGTT**TTAG**C TCAGATTCGG
AAGTGGTTGG ACAA

Fig. 1. Analysis of IKAP exon 20 sequence for ESS motifs. The 74 bp nucleotide sequence of IKAP was analyzed for putative ESS motifs. Two were found, each matching a reported ESS consensus sequence, PyTAG, and are presented in bold.

EGCG on the amount of alternatively spliced hnRNP A2 and B1 transcripts present in FD-derived fibroblast cells. For this study, RNA isolated from GM04663 cells treated for 24 h with varying concentrations of EGCG was subjected to real-time RT-PCR analysis using a pair of primers that recognize both of the alternatively spliced hnRNP A2 and B1 transcripts. A clear concentration-dependent decrease in the level of this transcript was observed (Fig. 2). Primers that recognized the A2 and B1 transcripts independently gave identical results (data not shown). This finding prompted an examination of the effect of EGCG on the cellular level of the exon 20-containing IKAP mRNA. For this study, FD-derived and normal fibroblasts were treated with 50 μg/ml EGCG for varying lengths of time and levels of hnRNP A2/B1, hnRNP A1, IKAP exon 20-containing (wild-type), IKAP exon 20-lacking (mutant), and IKAP exon 34–35-containing (total) transcripts were determined (Fig. 3). EGCG treatment results in a time-dependent reduction in the level of the hnRNP A2/B1transcript while having no effect on the level of the hnRNP A1 transcript. The absence of an impact on hnRNP A1 levels revealed that EGCG does not have a generalized effect on all members of the hnRNP A/B family. Exon 20-containing IKAP transcript was elevated in FD-derived cells but not in normal cells. The elevated presence of this transcript appears to be due to a modulation of the splicing process and not an increase in transcription, as the level of the exon 20-lacking transcript was not affected by EGCG and normal cells failed to exhibit an increase in the level of the exon 20-containing transcript. Interestingly, measurement of the amount of exon 34- and 35-containing IKAP, as a measure of total IKAP transcript present, reveals that EGCG treatment of FD-derived cells results in an increase in the cellular presence of the IKAP transcript. This observation is perhaps not surprising as, in contrast to the incorrectly processed transcript which contains a premature termination codon and is degraded by the nonsense-mediated mRNA decay pathway [27], the correctly processed IKAP transcript would be stable. The effect of EGCG treatment was further monitored by RT-PCR analysis using primers located in exons 19 and 23 capable of amplifying both the normal and mutant forms of the IKAP transcript. A clear enhanced presence of exon 20-containing transcript is present in the EGCG treated FD-derived cells while having no effect on the normal cells (Fig. 4).

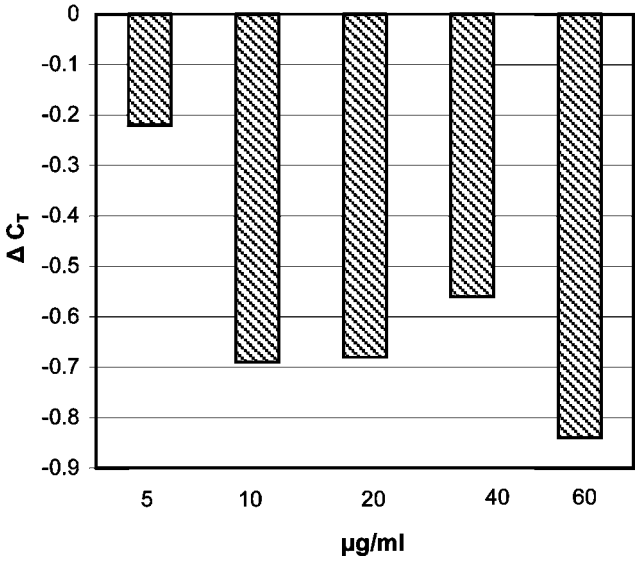

Fig. 2. Real-time RT-PCR analysis of hnRNP A2/B1 transcripts in EGCG-treated FD-derived GM04663 cells. Cultures were treated for 24 h with varying concentrations of EGCG. The relative amounts of hnRNP A2/B1 RNA were determined by real-time RT-PCR and are presented as changes in the threshold cycle (ΔC_T) relative to RNA levels from untreated cells. Results presented represent mean values obtained in three experiments, each done in triplicate.

Western blot analysis using antibodies recognizing hnRNP A1 and hnRNP A2/B1 confirms the ability of EGCG treatment to suppress the level of hnRNP A2/B1 while hnRNP A1 levels are unaffected (Fig. 5). Antibody to IKAP that recognizes the full-length protein was used to probe Western blots on which extracts of EGCG-treated GM00850 and GM0446 cells were fractionated. A clear increase in the amount of IKAP present in FD-derived fibroblasts was detected (Fig. 5).

The recently observed ability of tocotrienols to elevate transcription of *IKBKAP* prompted an evaluation of the response of FD-derived cells to the combined treatment of tocotrienols and EGCG. For this study, cells were treated with a combination of δ-tocotrienol and EGCG, at doses that by themselves did not result in a significant elevation in the level of the wild-type IKAP transcript, and the levels of the exon 20-containing IKAP transcript and full-length IKAP protein were determined. A clear synergistic increase in the level of the transcript and protein was observed (Fig. 6). Similar results were obtained with the GM00850, FD-derived cell line (data not shown).

Discussion

Mutations located in non-coding regions, such as those affecting 5′ and 3′ splice sites, branch sites and

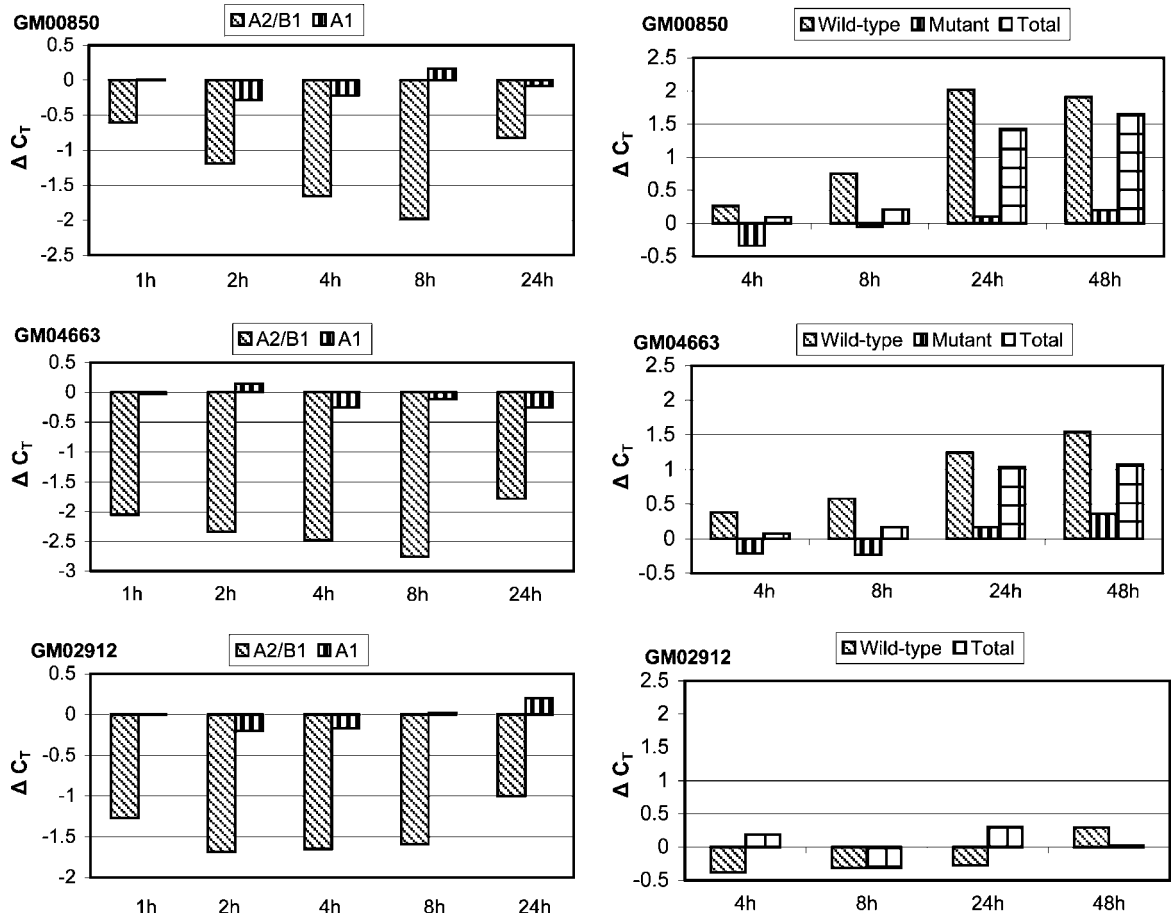

Fig. 3. Real-time RT-PCR analysis of hnRNP A2/B1, hnRNP A1, and IKAP RNA levels in EGCG-treated cells. GM00850 (FD-derived), GM04663 (FD-derived), and GM02912 (normal) cells were treated for varying times with 50 μg/ml EGCG. The relative amounts of hnRNP A2/B1, hnRNP A1, wild-type IKAP (exon 20-containing), mutant IKAP (exon 20-lacking), and total IKAP (exon 34–35) RNA were determined by real-time RT-PCR and are presented as changes in the threshold cycle (ΔC_T) relative to RNA levels from untreated cells. The panels on the left show changes in hnRNP RNA levels and those on the right, changes in IKAP RNA levels. Results presented represent mean values obtained in three experiments, each done in triplicate.

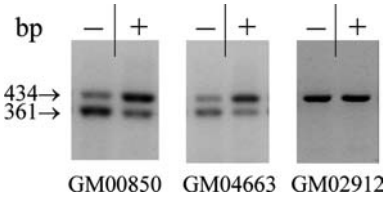

Fig. 4. RT-PCR analysis of wild-type (exon 20-containing) and mutant (exon 20-lacking) IKAP transcripts in EGCG-treated cells. GM00850 (FD-derived), GM04663 (FD-derived), and GM02912 (normal) cells were treated for 24 h with 50 μg/ml EGCG. The relative amounts of wild-type (434 nt) and mutant (361 nt) IKAP transcripts were determined in RT-PCR reactions with primers spanning exons 19–23. The products were analyzed on 2% agarose gels. Results presented represent a typical result obtained.

polyadenylation signals are often the cause of hereditary diseases [28]. Approximately 15% of the single base mutations that cause human genetic diseases result in RNA splicing defects [29]. Some of these mutations result in an apparent absence of any functional gene product while others allow some functional gene product to be produced. These latter "leaky" mutations often result in milder genetic disorders or disorders that manifest themselves later in life [30–34]. The ability of the splicing machinery to modulate the impact of genetic alterations has led to the development of approaches that yield increasing amount of correctly spliced transcripts. These approaches include the use of antisense oligonucleotides that target splice-site motifs and thereby alter splicing efficiencies [35] or the over-expression of splicing factors that facilitate the production of the correctly spliced transcript. This increased expression of splicing factors has been accomplished via transfection [10,36,37] or through the use of chemical compounds [38] that modulate the cellular content of such proteins.

The presence of putative ESS elements in exon 20 of *IKBKAP*, the ability of hnRNPs to bind ESSs and thereby suppress splicing and promote intron-distal 5′ splice site utilization [5–7], and the existence of a

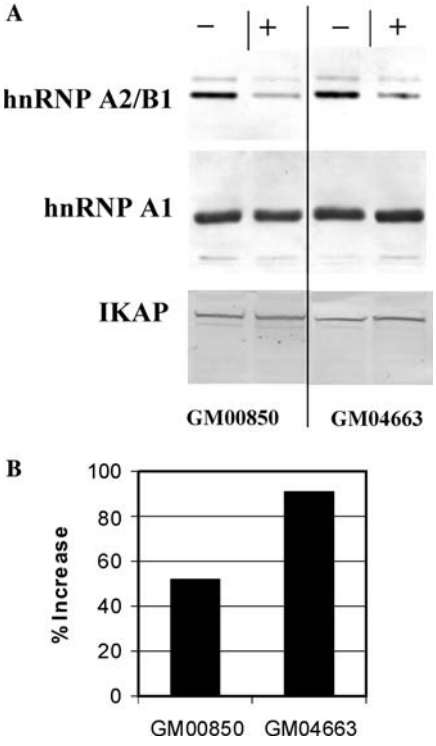

Fig. 5. Western blot analysis of hnRNP A2/B1, hnRNP A1, and IKAP levels in EGCG-treated cells. Two FD-derived cell lines, GM00850 and GM04663, were treated for 24 h with 50 μg/ml EGCG. Protein extracts were fractionated by SDS–PAGE, blotted onto nitrocellulose, and probed with antibodies against hnRNP A2/B1, hnRNP A1, and IKAP. Shown in (A) are results obtained in untreated (−) and EGCG-treated (+) cells. The blots were analyzed densitometrically to determine the % increase in the amounts of IKAP produced by EGCG treatment relative to untreated cells (B). Results presented represent a typical result obtained.

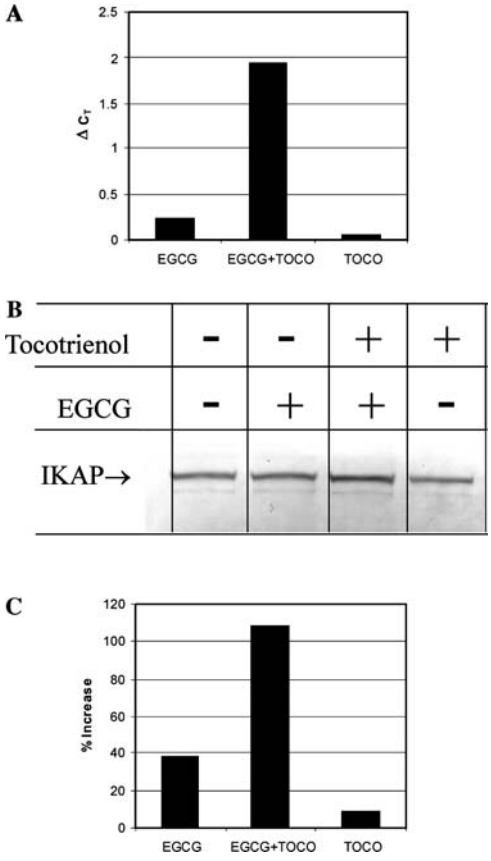

Fig. 6. Real-time and Western blot analysis of IKAP levels in EGCG and tocotrienol treated cells. The FD-derived cell line, GM04663, was treated with 5 μg/ml EGCG, 6.25 μg/ml δ-tocotrienol or a combination of the two for 24 h. The relative amounts of exon 20-containing (wild-type) RNA were determined by real-time RT-PCR and are presented as changes in the threshold cycle (ΔC_T) relative to RNA levels from untreated cells (A). Results presented represent mean values obtained in three experiments, each done in triplicate. Protein extracts were subjected to Western blot analysis and probed with IKAP antibody (B). Results presented represent a typical result obtained. The blots were analyzed densitometrically to determine the % increase in the amounts of IKAP produced by the various treatments relative to untreated cells (C).

weakened 5′ splice site due to the FD-causative mutation, suggested that a reduction in the level of hnRNPs might increase the production of the exon 20-containing IKAP transcript. The ability of EGCG treatment of cells to reduce the levels of hnRNP B1 protein and hnRNP A2/B1 gene promoter activity [23] prompted an examination of the impact of EGCG treatment on the expression of IKAP in FD-derived and normal fibroblast cells. We report that EGCG treatment results in a decrease in the cellular levels of the hnRNP A2/B1 transcript and protein while having no effect on the level of hnRNP A1. EGCG treatment of FD-derived cells results in an increase in the level of the exon 20-containing IKAP transcript, has no appreciable effect on the level of the exon 20-lacking transcript, increases the overall amount of the IKAP mRNA as measured by the amount of exon 34–35-containing transcript, and increases the amount of IKAP protein present in these cells. The amount of exon 20- or exon 34–35-containing transcripts is unaffected in normal cells. The increase in the exon 20-containing, but not the exon 20-lacking, transcript and the absence of any modulation of IKAP transcript levels in normal cells suggest that EGCG is impacting the splicing of the IKAP transcript rather than the rate of transcription. These observations suggest that the reduction in the cellular level of hnRNPA2/B1 may allow for the increased use of the intron-proximal 5′ splice site, resulting in an increased presence of the exon 20-containing transcript. The increase in the amount of IKAP transcript containing exons 34–35 in the EGCG-treated cells likely reflects the stability of the correctly spliced transcript which lacks the premature stop codon present in the abnormally spliced transcript and is therefore not a target of the nonsense-mediated mRNA decay pathway [27].

As FD affects the development and survival of sensory, sympathetic, and parasympathetic neurons, we examined the impact of EGCG treatment on hnRNP

A2/B1 levels in the neuroblastoma-derived LA1-55n cell line which has characteristics of sympathetic neurons [39,40] and observed a dose-dependent reduction in the level of hnRNP A2/B1 (data not shown). Fujimoto et al. [23] reported that genistein, like EGCG, reduces the level of hnRNP B1 in A549 cells. We observed that genistein reduces the level of hnRNP A2/B1 transcript and increases the production of functional IKAP in FD-derived cells, though the kinetics of the response differs from that of EGCG-treated cells (data not shown).

The ability of tocotrienols to elevate the level of transcription of the *IKBKAP* gene [21] prompted an investigation of the combined impact of EGCG and tocotrienols on the amount of exon 20-containing IKAP transcript and IKAP protein produced in FD-derived cells. We report that the combination of doses of these agents that by themselves fail to substantially increase the level of the exon 20-containing transcript and IKAP protein results in a significantly increased level of transcript and protein.

EGCG is a major polyphenolic antioxidant present in green tea that has been reported to block carcinogenesis, inhibit the growth and induce apoptosis of cancer cells, modulate gene expression, and possess anti-microbial activity against bacteria, fungi, and viruses [41–47]. The mechanisms by which EGCG mediates these effects continue to be elucidated. In this report, we demonstrate the ability of EGCG to increase the production of functional IKAP in FD-derived cells and suggest that the downregulation of hnRNP A2/B1 may be responsible for this event. As we also observed a reduction in hnRNP A2/B1 in neuronal cells, the implication is that EGCG has the potential to positively impact IKAP levels in neurons of FD patients. We therefore propose that EGCG supplementation in individuals with FD may result in an in vivo modulation of splicing that results in the production of elevated levels of functional IKAP mRNA and protein. Furthermore, the observed synergism between the actions of the tocotrienols and EGCG suggests that individuals with FD may benefit from their combined use.

Acknowledgments

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