The nonsense-mediated RNA decay pathway is disrupted in inflammatory myofibroblastic tumors

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Inflammatory myofibroblastic tumors (IMTs) are characterized by myofibroblast proliferation and an inflammatory cell infiltrate. Little is known about the molecular pathways that precipitate IMT formation. Here, we report the identification of somatic mutations in \textit{UPF1}, a gene that encodes an essential component of the nonsense-mediated RNA decay (NMD) pathway, in 13 of 15 pulmonary IMT samples. The majority of mutations occurred in a specific region of \textit{UPF1} and triggered \textit{UPF1} alternative splicing. Several mRNA targets of the NMD pathway were upregulated in IMT samples, indicating that the \textit{UPF1} mutations led to reduced NMD magnitude. These upregulated NMD targets included \textit{NIK} mRNA, which encodes a potent activator of NF-κB. In human lung cells, UPF1 depletion increased expression of chemokine-encoding genes in a NIK-dependent manner. Elevated chemokines and IgE class switching events were observed in IMT samples, consistent with NIK upregulation in these tumors. Together, these results support a model in which \textit{UPF1} mutations downregulate NMD, leading to NIK-dependent NF-κB induction, which contributes to the immune infiltration that is characteristic of IMTs. The molecular link between the NMD pathway and IMTs has implications for the diagnosis and treatment of these tumors.

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Inflammatory myofibroblastic tumors (IMTs) are characterized by myofibroblast proliferation and an inflammatory cell infiltrate. Little is known about the molecular pathways that precipitate IMT formation. Here, we report the identification of somatic mutations in UPF1, a gene that encodes an essential component of the nonsense-mediated RNA decay (NMD) pathway, in 13 of 15 pulmonary IMT samples. The majority of mutations occurred in a specific region of UPF1 and triggered UPF1 alternative splicing. Several mRNA targets of the NMD pathway were upregulated in IMT samples, indicating that the UPF1 mutations led to reduced NMD magnitude. These upregulated NMD targets included NIK mRNA, which encodes a potent activator of NF-κB. In human lung cells, UPF1 depletion increased expression of chemokine-encoding genes in a NIK-dependent manner. Elevated chemokines and IgE class switching events were observed in IMT samples, consistent with NIK upregulation in these tumors. Together, these results support a model in which UPF1 mutations downregulate NMD, leading to NIK-dependent NF-κB induction, which contributes to the immune infiltration that is characteristic of IMTs. The molecular link between the NMD pathway and IMTs has implications for the diagnosis and treatment of these tumors.

Results and Discussion

Somatic UPF1 mutations in IMTs elicit exon skipping. Based on our previous identification of frequent UPF1 mutations in pancreatic tumors (5), we screened IMT lung samples to determine whether they also had UPF1 mutations. Sequence analysis of UPF1 revealed mutations in IMTs from 13 of 15 patients (Supplemental Table 1 and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI86508DS1). In total, 41 mutations were detected. All but one of these 41 mutations were clustered at 11 sites in exon 10 and intron 10 (Supplemental Figure 1B). Mutations at 8 of these 11 sites were found in more than one tumor (Supplemental Figure 1B). The mutations in the 13 IMTs were not detected in disease-free lung tissues and thus were somatic in origin (data not shown).

UPF1 exon 10 and intron 10 are unusually short (169 and 85 nt, respectively) and have a high GC content (59% and 73%, respectively), both of which are features that can weaken RNA splicing (6). This raised the possibility that intron 10 splicing depends on local splicing enhancers (7) and that these elements are disrupted by the mutations we identified in the UPF1 gene, leading to aberrant UPF1 splicing. To test this possibility, we constructed a UPF1 minigene containing the relevant region of UPF1 (Figure 1A). When transfected into HEK293 cells, this minigene was normally spliced, as shown by direct sequencing of the only band observed by RT-PCR analysis (Figure 1B). We then introduced the mutations found in IMTs (Figure 1A and Supplemental Figure 2A). This caused reduced expression of the normally spliced mRNA and the appearance of an alternatively spliced transcript lacking exons 10 and 11 (Figure 1B and Supplemental Figure 2B).
revealed that it was expressed at a significantly lower steady-state RNA level than was the full-length UPF1 construct (Figure 1E), an effect that was mirrored at the protein level (Figure 1E). The simplest explanation for these findings is that the loss of exons 10 and 11 destabilizes UPF1 RNA.

As an independent means to analyze UPF1 protein expression levels, we examined IMT tissue sections, which unlike NT, had not only alveolar epithelial cells but also inflammatory cells (Figure 1, F and G, and Supplemental Figure 3), as is characteristic of IMTs (1, 3). Immunohistochemical (IHC) analysis with a highly specific UPF1 antibody showed that normal alveolar epithelial cells expressed

To examine endogenous UPF1 splicing, we analyzed the IMT tumor from patient 15, which was frozen, providing sufficiently high quality RNA for analysis. RT-PCR analysis revealed an alternatively spliced UPF1 transcript identical to that generated by the mutant minigene in IMT tissue (Figure 1C). This alternatively spliced UPF1 transcript was not present in normal lung tissue (NT) from the same patient. Consistent with the reduced level of UPF1 mRNA in IMT (Figure 1C), the UPF1 protein level was also reduced (Figure 1D). To investigate the mechanism, we generated a UPF1 cDNA construct lacking exons 10 and 11. Transfection of this mutant construct into HEK cells depleted of endogenous UPF1 revealed that it was expressed at a significantly lower steady-state RNA level than was the full-length UPF1 construct (Figure 1E), an effect that was mirrored at the protein level (Figure 1E). The simplest explanation for these findings is that the loss of exons 10 and 11 destabilizes UPF1 RNA.

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high levels of UPF1, while most patient IMT alveolar epithelial cells exhibited little or no UPF1 expression (Figure 1, H–J, Supplemental Figure 3, and Supplemental Table 2). The only exceptions were the epithelial cells in the two IMTs lacking UPF1 mutations and the IMT with a missense UPF1 mutation (in exon 13), all of which expressed high levels of UPF1 (Supplemental Figure 3). In contrast with the alveolar epithelial cells, the inflammatory cells in IMTs had high UPF1 antibody reactivity regardless of UPF1 mutation status (Supplemental Figure 3 and Supplemental Table 2). These findings suggest that UPF1 mutations occur specifically in the alveolar epithelial cells in IMTs, leading to reduced UPF1 protein levels in these cells.

We next examined the functional consequences of the single UPF1 mutation we identified outside of the exon 10-11 region (c.1796A>T p.K599M from patient 13; Supplemental Table 1). The strong UPF1 IHC staining in this patient sample (Supplemental Table 2) suggested that this mutation does not destabilize UPF1 RNA, and raised the alternative possibility that it disrupts UPF1 activity. In support, we found that exogenous UPF1 harboring the missense mutation corresponding to patient 13 failed to rescue NMD activity in UPF1-depleted cells (as it did not significantly decrease the level of an NMD-sensitive NS39 β-globin mRNA), indicating that this mutant lacked detectable NMD function. Furthermore, this mutant conferred dominant-negative activity, as it reduced the activity of exogenous WT UPF1 (Supplemental Figure 4). Together with our analysis of the other UPF1 mutants described above, this suggested that two types of mutations are employed to reduce UPF1 activity in IMTs: those that generate a dominant-negative UPF1 or an unstable form of UPF1 mRNA.

IMTs have disrupted NMD and elevated expression of the proinflammatory molecule NIK. The dramatically reduced level of UPF1 expression in most IMTs raised the possibility that NMD is depressed in IMTs, leading to upregulation of NMD target transcripts. In agreement with this hypothesis, we found that 11 endogenous NMD substrates were upregulated in the patient 15 IMT sample relative to NT from the same patient (Supplemental Table 3). We next considered that perturbed NMD would allow mRNAs encoding proinflammatory proteins to be stabilized, leading to the immune infiltration characteristic of IMTs. We identified mitogen-activated protein kinase kinase kinase 14 (MAP3K14 or NIK) as a good candidate, as it is an activator of the proinflammatory NF-κB pathway.
In support of this, insertion of the NIK (9, 10), and so we assessed the mechanism by which NIK an NMD substrate by virtue of its possessing a uORF. NIK in BEAS-2B cells was confirmed at the protein level (Figure 2B, lower) and in IMT tissue harboring NIK mutations had upregulated NIK protein expression (Supplemental Table 2). Upregulated NIK expression was specific to lung tumor cells, as the local infiltrating leukocytes had low/no NIK expression in all patients (Supplemental Figure 5 and Supplemental Table 2). The upstream ORF (uORF) present in the NIK mRNA is responsible for it being degraded by NMD. Stop codons terminating short ORFs upstream of the protein-coding ORF can elicit NMD (4). Thus, we considered the possibility that the upstream ORF (uORF) present in the 5′ UTR of NIK mRNA is responsible for it being degraded by NMD. In support of this, insertion of the NIK 5′ UTR in front of a luciferase (Luc) reporter (Figure 2F) led to increased reporter expression in UPF1-depleted cells, as measured at the RNA level or by Luc activity (Figure 2G). Mutation of the uORF start ATG by replacing in UPF1-depleted cells, as measured at the RNA level or by Luc reporter (Figure 2F) led to increased reporter expression. Using an RT-PCR assay, we observed a 0.5-kb band indicative of germline transcription (12, 13). The commitment of a B cell to isotype class switch to an IgE-producing cell requires induction of Cε germline transcription (14, 15). We thus assessed whether IMTs have upregulated germline transcription, which we confirmed was a germline transcript (exons 1 and 3; 487 bp). GAPDH is the loading control. The lanes were run on the same gel but were noncontiguous. (E and F) IHC analysis of patient 15 (×200). The red arrow, IgE-positive cell (n = 3).

In the IMT tissue had the morphology of plasma or mast cells. In contrast, neither of the two IMT tumors lacking detectable UPF1 mutations had upregulated NIK protein expression (Supplemental Table 2). Upregulated NIK expression was specific to lung tumor cells, as the local infiltrating leukocytes had low/no NIK expression in all patients (Supplemental Figure 5 and Supplemental Table 2). NIK mRNA was previously suggested to be an NMD substrate (9, 10), and so we assessed the mechanism by which NIK mRNA is degraded by NMD. Stop codons terminating short ORFs upstream of the protein-coding ORF can elicit NMD (4). Thus, we considered the possibility that the upstream ORF (uORF) present in the 5′ UTR of NIK mRNA is responsible for it being degraded by NMD. In support of this, insertion of the NIK 5′ UTR in front of a luciferase (Luc) reporter (Figure 2F) led to increased reporter expression in UPF1-depleted cells, as measured at the RNA level or by Luc activity (Figure 2G). Mutation of the uORF start ATG by replacing in UPF1-depleted cells, as measured at the RNA level or by Luc reporter (Figure 2F) led to increased reporter expression. Using an RT-PCR assay, we observed a 0.5-kb band indicative of germline transcription (12, 13). The commitment of a B cell to isotype class switch to an IgE-producing cell requires induction of Cε germline transcription (14, 15). We thus assessed whether IMTs have upregulated germline Cε transcripts. Using an RT-PCR assay, we observed a 0.5-kb band indicative of Cε transcription, which we confirmed was a germline Cε transcript by direct sequencing (Figure 3D). Consistent with activation of Cε transcription and rearrangement, IHC analysis showed that patient 15 had many IgE-positive cells (Figure 3E), whereas normal lung tissue from the same patient did not (Figure 3F). The IgE-positive cells in the IMT tissue had the morphology of plasma or mast cells.

Stabilized NIK elevated chemokine gene expression and IgE production. NIK is a serine/threonine protein kinase that stimulates NF-κB activity (8). Given that a hallmark of NF-κB activation is the generation of chemokines (11), this raised the possibility that the upregulation of NIK in IMTs has a causal role in these inflammatory tumors. In support of this possibility, we found that UPF1 depletion dramatically increased IL-8, CCL20, and CXCL1 mRNA expression in the BEAS-2B lung cell line (Figure 3A). This upregulated chemokine expression resulted, at least in part, from upregulated NIK expression, as it was largely abolished when we prevented NIK upregulation using RNAi (Figure 3A). Together, these data support a model in which NMD normally serves to suppress the chemokine response by destabilizing NIK mRNA (Figure 3B). When NMD is perturbed, this suppression is lifted, leading to high NIK expression and the generation of high levels of chemokines, thereby triggering immune cell infiltration, the hallmark of IMTs (1). As evidence that this model applies in vivo, RT-qPCR analysis revealed upregulated expression of the chemokine genes IL-8, CCL20, and CXCL1 in the patient 15 IMT relative to NT (Figure 3C).

Another property of NIK is its ability to promote B cell proliferation and induce B cells to differentiate into IgE-secreting plasma cells (12, 13). The commitment of a B cell to isotype class switch to an IgE-producing cell requires induction of Cε germline transcription (14, 15). We thus assessed whether IMTs have upregulated germline Cε transcripts. Using an RT-PCR assay, we observed a 0.5-kb band indicative of Cε transcription, which we confirmed was a germline Cε transcript by direct sequencing (Figure 3D). Consistent with activation of Cε transcription and rearrangement, IHC analysis showed that patient 15 had many IgE-positive cells (Figure 3E), whereas normal lung tissue from the same patient did not (Figure 3F).
Our data support a model in which NMD normally serves to confer tight regulation on NIK abundance in order to place cells below the threshold required for NF-κB pathway induction and inappropriate immune activation. By analogy, NMD has been shown to raise the threshold for triggering the unfolded protein response, thereby preventing programmed cell death in response to innocuous stimuli (16, 17). Interestingly, the vast majority of the mutations that we observed in IMTs were clustered in one of the two same regions of the UPF1 gene as in adenosquamous pancreatic cancer (the exon 10-intron 10-exon 11 region; Supplemental Figure 1B). Indeed, 7 of the pulmonary IMTs had UPF1 mutations at precisely the same site as in some ASC tumors (IVS10+32C>T and IVS10-25C>T). We speculate that the aberrations in NMD caused by genetic alterations in the UPF1 gene lead to different disease outcomes, depending on the cell type. In premalignant glandular pancreatic cells, this may lead to reprogramming towards a squamous, more malignant state (S). In lung epithelial cells, NMD perturbation may drive a proinflammatory response, as suggested by our results herein.

Our finding that UPF1 mutations are a common molecular alteration associated with IMTs suggest that UPF1 mutations and NMD activity may be useful diagnostic markers for this disease. New diagnostic markers are critical, as IMTs are often misdiagnosed as malignant tumors (1, 2). Further, our results described herein suggests that therapies directed at restoring the NMD pathway and NIK expression have the potential to ameliorate or cure the symptoms of IMT and related diseases, including IgG4-related disease (IgG4-RD), an autoimmune disorder that resembles IMTs (1, 3). New therapies are desired, as current methods to treat IMTs and IgG4-RD, including surgery and antiinflammatory agents, are (1, 3). New therapies are desired, as current methods to treat IMTs and IgG4-RD, including surgery and antiinflammatory agents, are invasive or have undesirable side effects (2, 3).

Methods

Subjects. We evaluated a set of IMTs from 15 patients, provided by the First Affiliated Hospital of Bengbu Medical College. Among them, 14 specimens were formalin-fixed paraffin-embedded (FFPE) tissue sections, and one IMT of patient 15 was frozen immediately after collection. All patients provided written informed consent.

Statistics. The program ESE Finder was used to locate several predicted ESEs and ISEs in the regions of the UPF1 gene. Pairwise comparisons were performed by 2-tailed Student’s t test using Excel software (Microsoft). Data are expressed as the mean ± SEM.

Study approval. All aspects of the study were approved by the Bengbu Medical College and Tongji University School of Medicine.

For DNA/RNA sequencing and analysis, constructs, cell culture and transfections, and protein analysis, see Supplemental Methods.

Author contributions

CL, WY, GY, and TP performed UPF1 screening. JL and AH constructed wild-type and mutant mini-gene expression vectors. JL, FS, SL, and YW performed IHC, Western, and RNA analyses. FS, CS, VJ, and CY provided pathology samples, clinical data, and expertise on IMTs. JL and TP prepared figures and tables and wrote the manuscript. YL and MFW designed and supervised the project and assisted in writing the manuscript.

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