a phase 2 clinical trial with a combination of the SYK inhibitor GS9973 and idelalisib, 13.6% of CLL and/or non-Hodgkin lymphoma patients developed severe pneumonitis leading to early termination of the study.[15] The underlying pathophysiology of how signal transduction inhibitors are associated with pulmonary toxicities remains to be defined.

Given the findings of a nonmalignant inflammatory infiltrate, we hypothesize that inhibiting signal transduction pathways enhances expression of proinflammatory cytokines and the innate immune system. BTK also appears to serve as a critical mediator of lipopolysaccharide-induced dendritic cell maturation and macrophage polarization. Several studies have reported an increase in alveolar infiltration of T helper 2 proinflammatory cytokines in BTK-deficient mice, resulting in airway inflammation.[16,17]

Our findings have important clinical implications. We propose that patients may receive counseling about this potential toxicity prior to initiating ibrutinib therapy and that any respiratory illness be taken seriously and evaluated effectively. Management strategies could include dose interruption and/or drug discontinuation along with initiation of steroids in severe cases. Further studies and better understanding of this toxicity are needed as the use of ibrutinib for treating lymphoid malignancies is expected to increase in the near future.

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To the editor:

Human *STEAP3* mutations with no phenotypic red cell changes

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During recent years, variants in iron metabolism genes have been identified as molecular etiologic factors of hereditary microcytic anemias, such as iron-refractory iron deficiency anemia caused by *TMPRSS6* mutations, hypotransferrinemia resulting from *TF* mutations,
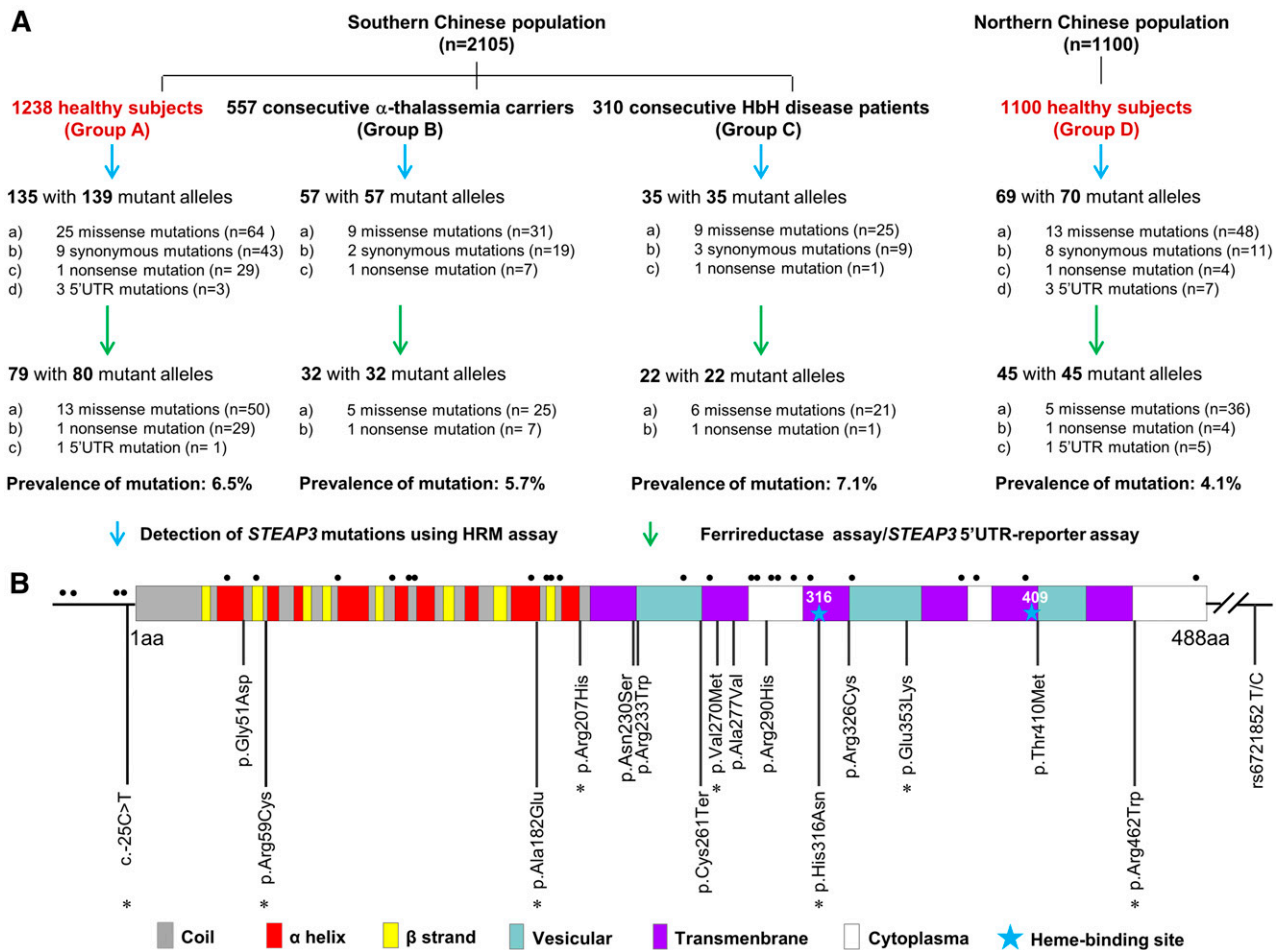
Figure 1. The prevalence and spectrum of *STEAP3* mutations in the Chinese population. (A) Study design and study outcomes. A total of 3205 individuals from 2 representative regions (southern and northern China) were studied to document the incidence of *STEAP3* mutations. Southern Chinese population: 3 groups of 2105 subjects from Guangxi where thalassemias are endemic included 1238 normal subjects (group A), 557 α-thalassemia carriers (group B), and 310 individuals with hemoglobin H (HbH) disease samples (group C). *STEAP3* mutations occurred at similar frequencies among these 3 groups (*P* = .720), confirming a high prevalence of *STEAP3* mutations in southern China. The high prevalence of *STEAP3* mutations was also noted in 1100 normal individuals recruited from the Shandong Province in northern China. All 179 mutant alleles detected in these 2 populations were used to assess the spectrum of *STEAP3* mutations. (B) Domains in the gene structure of STEAP3 are indicated by the different color boxes and the untranslated regions are delineated by the solid black line. Sixteen functional *STEAP3* mutations are shown below the map and 8 novel mutations are marked with an asterisk. Twenty-eight neutral mutations (4 variants in the 5′UTR and 23 missense mutations in the coding regions) are shown with black dots above the map and are presented in supplemental Table 1. The eSNP (rs6721852) in the *STEAP3-C2orf76* intergenic region is also indicated at the right.

congenital sideroblastic anemia (CSA) due to mutations in *SLC25A38*, and so on.[1-3] Recently, the *STEAP3* gene, also known as *TSAP6*, was identified as one of the potential candidate for CSA, because it encodes an endosomal ferrireductase required for efficient transferrin-dependent iron uptake in developing erythroblasts.[4-6] *Steap3*/Tsap6 null mice display moderately severe microcytic anemia due to reduced activity of ferrireductase and abnormal erythroid maturation.[5,7-9] Recently, the first, and thus far only, human *STEAP3* mutation causing transfusion-dependent severe hypochromic anemia was reported in 1 family due to a combination of a mutant allele (p.Cys100Ter) and a hypomorphic allele.[10]

Our study investigated the prevalence of *STEAP3* mutations in humans and their physiologic consequences. We used large cohorts of normal individuals and α-thalassemia subjects from China to study the phenotypic effect of *STEAP3* mutations on normal and abnormal erythrocyte indices. Surprisingly, we found a relatively high prevalence of potentially harmful recessive alleles. However, although the identified *STEAP3* mutations exhibited impaired ferrireductase activity in vitro, they had little or no effect on erythrocyte phenotypes.

Details regarding the study design and methods used are available in the supplemental Data available on the *Blood* Web site. In the present population-based study, using a molecular screening approach (supplemental Figure 1) and functional analysis, we found multiple mutations in *STEAP3* with a surprisingly high prevalence of 5.3% in a total of 2338 Chinese healthy subjects, with slightly higher rates in southern China (6.5%) than in northern China (4.1%, *P* = .011; Figure 1A). A total of 179 mutant alleles involving 16 different loss-of-function *STEAP3* mutations (14 missense mutations, 1 nonsense mutation, and 1 mutation in the 5′ untranslated region [UTR]) were identified in these 2 geographically distinct study populations (Figure 1B), each with their own characteristic spectrum of mutations. The 3 most common mutations, p.Cys261Ter, p.Arg290His, and p.Gly51Asp, accounted for 72.6% of the total (supplemental Table 1). As shown in Figure 1B and supplemental Figure 2, the affected residues within the key domains of the protein are highly conserved evolutionarily and may be critical for enzyme activity.

We systematically performed molecular and functional analysis of all variants on *STEAP3* and identified 16 different loss-of-function *STEAP3* mutations. As shown in Figure 2A, the various mutant
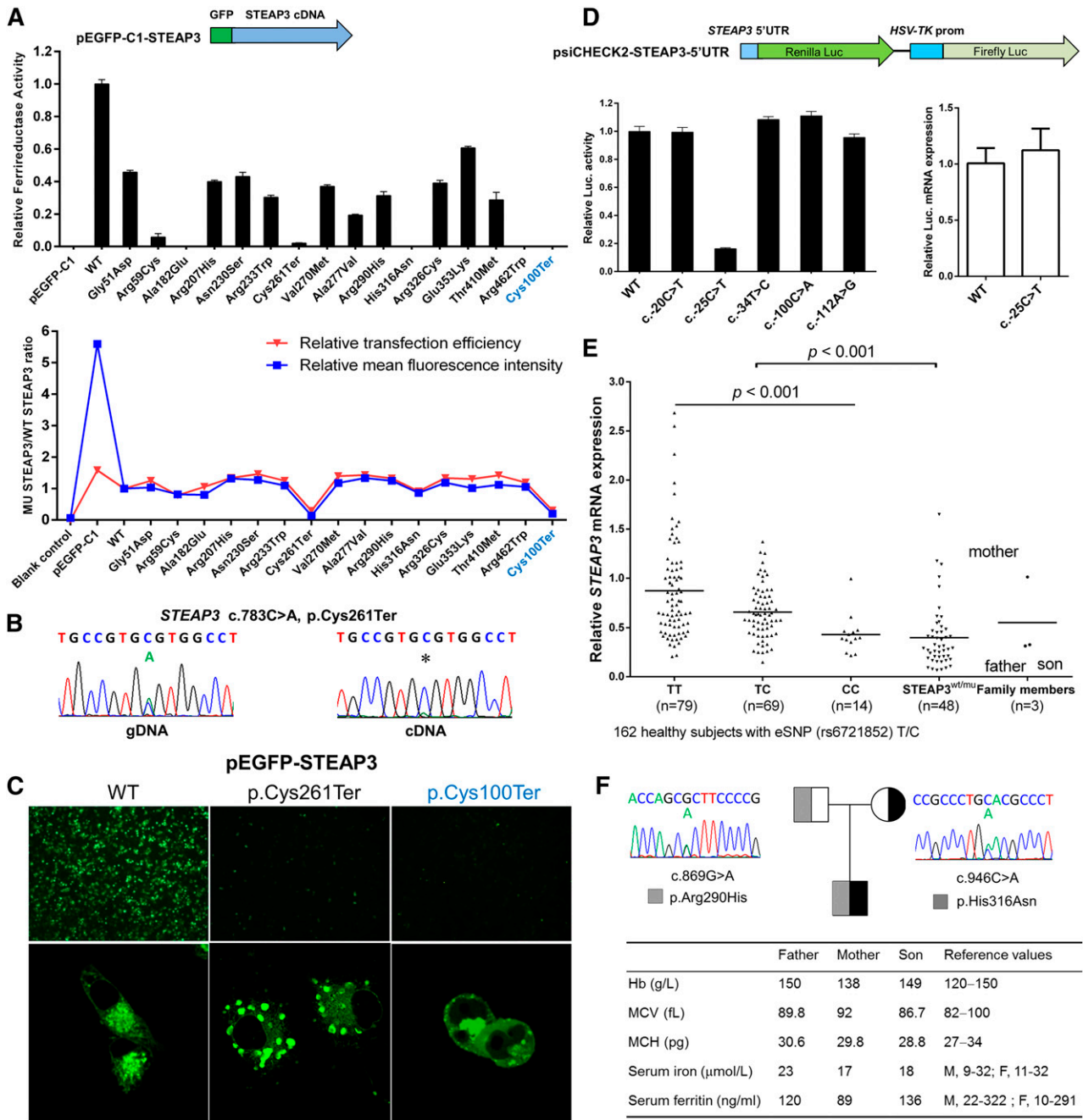
Figure 2. Effects of mutations on STEAP3 function. (A) Transient transfection of HeLa cells with GFP-tagged STEAP3 expression constructs of wild-type (WT) and mutant cDNA to determine relative ferrireductase activity by iron reductase assay. Five mutants completely abrogated this activity, whereas 10 expressed moderately decreased activity compared with the wild-type STEAP3. Similarly transfection efficiency and stable STEAP3 protein expression were noted for all mutant STEAP3s except for 2 nonsense mutants (p.Cys261Ter and p.Cys100Ter) by flow cytometry analysis. (B) Sequence analysis of genomic DNA and cDNA from a case with the *STEAP3* nonsense mutation (c.783C>A, p.Cys261Ter) indicated that the mutant allele was massively degraded but still could be detected at the cDNA level. (C) Subcellular localization of GFP-tagged wild-type STEAP3 and the 2 mutants in COS7 cells in images taken at ×4 and ×180 magnification. Confocal microscopy confirmed the endosomal and the plasma membrane localization of the wild-type protein, whereas the p.Cys261Ter mutant formed unusual aggregates, and the p.Cys100Ter mutant localized in both the nucleus and the cytoplasm of COS7 cells. Only a small number of cells exhibited these changes (bottom center/right figures), and most of cultured cells were not detected due to lack of fluorescence. (D) The *Renilla* luciferase gene was used as a reporter driven by the *STEAP3* 5′UTR with firefly luciferase as the endogenous control. The −25C>T mutant decreased STEAP3 expression at the protein level, whereas the mRNA levels remained relatively stable compared with the wild type. (E) Quantitative RT-PCR analysis of the *STEAP3* mRNA from peripheral blood cells. A cohort of 210 samples randomly selected from southern China was used for mRNA quantification, which included the healthy group (n = 162), consisting of 3 su-groups according to the eSNP allele at rs6721852 (n = 79 in T/T, n = 69 in T/C, and n = 14 in C/C) and the *STEAP3* mutant group (n = 48). Three members from a family with compound heterozygous mutations (p.His316Asn and p.Arg290His) are shown in the last column. (F) Study of a family with compound heterozygous *STEAP3* mutations. The hematologic parameters (Hb, mean cell volume [MCV], and mean cell hemoglobin [MCH]), serum iron, and serum ferritin are indicated in the table. The p.His316Asn is a severe mutation, whereas the p.Arg290His is a moderate mutation. Data in A, D, and E represent mean ± standard deviation of 3 independent experiments.

STEAP3 proteins exhibited impaired ferrireductase activity (5 severe and 10 moderately reduced). Notably, the p.Arg59Cys and the p.His316Asn mutations completely abrogated iron reduction, as mutations at these 2 sites are implicated in NAD(P)H-binding motif (Ser[58] and Arg[59]) or in heme-binding sites (His[316] and His[409]) of STEAP3.[5,6,11,12] Additionally, 2 other human STEAP3 mutants

(p.Ala182Glu and p.Arg462Trp) also severely impaired protein function, demonstrating the functional importance of these 2 residues. Surprisingly, the mutant messenger RNA (mRNA) (p.Cys261Ter), while massively degraded, could still be detected by complementary DNA (cDNA) sequencing (Figure 2B). It is likely that either incomplete nonsense-mediated decay or other mechanisms resulted in extremely low ferrireductase activity (Figure 2A) and protein expression in vitro (Figure 2A,C). Subcellular localization of this mutant green fluorescent protein (GFP)-tagged truncated protein revealed abnormal aggregates in the endosomes and on the plasma membrane (Figure 2C). However, all 14 missense mutations had no effect on protein expression (Figure 2A) and exhibited normal cellular localization (data not shown). Figure 2D demonstrates that the luciferase activity was significantly reduced by >80% for the mutant (c.-25C>T), whereas the mRNA level remained relatively stable, suggesting that the mutation in the *STEAP3* 5′UTR disrupts posttranscriptional regulation. We further measured *STEAP3* mRNA levels in human peripheral blood cells by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in Figure 2E, considerable variability and a wide range of *STEAP3* mRNA expression were observed in the 162 normal individuals, and significantly lower expression levels of *STEAP3* were noted in normal samples containing the C allele compared with the T allele at rs6721852 ($P < .001$), which is the expressed single nucleotide polymorphism (eSNP) in the *STEAP3-C2orf76* intergenic region (http://www.gtexportal.org/home/gene/STEAP3).[13] The expression levels were lower in all 48 subjects with *STEAP3* mutations compared with the 162 normal samples ($P < .001$). These findings confirm a previous report that *STEAP3* is expressed as a quantitative trait locus.[10,13,14]

Unexpectedly, we observed that there were no significant alterations in erythrocyte hematologic phenotypes in all subjects carrying those *STEAP3* heterozygous mutations (79 in group A and 45 in group D; Figure 1A), including 33 individuals with p.Cys261Ter variants (29 in group A and 4 in group D; Figure 1A), as shown in supplemental Table 2. Hypochromic anemia was not found in subjects with *STEAP3* mutations or normal individuals with low-level *STEAP3* mRNA expression due to the hypomorphic C allele. In addition to this, we also confirmed no changes in the 2 iron metabolic indices, serum iron and serum ferritin, in the individuals with 8 representative heterozygous mutations in *STEAP3* (supplemental Table 3). Interestingly, we identified a family (Figure 2F) in which the child carried compound heterozygous mutations (p.His316Asn, a null mutation from the father and p.Arg290His from the mother). There was decreased expression of *STEAP3* expression in both the father and the child (Figure 2E). Importantly, the hematologic parameters and iron status were essentially normal despite the compound heterozygosity (Figure 2F). This finding is in marked contrast to a recent report that described a transfusion-dependent severe hypochromic anemia in a family due to a combination of a mutant allele (p.Cys100Ter) and a hypomorphic allele.[10] Taking together, these findings suggest that only a very large reduction in enzyme activity or complete absence of activity could lead to the manifestation of the hypochromic erythrocyte phenotypes as suggested by Grandchamp et al.[10] Homozygous null *STEAP3* mutations such as the most common *STEAP3* null variant, p.Cys261Ter, would be sufficient to cause moderately severe congenital hypochromic anemia arising from lack of heme synthesis. However, we were unable to identify such cases in our study. Another possibility is that other pathways of reducing iron in the transferrin-cycle endosome are present in erythroid cells, as evidenced by the previous findings that *Steap3*$^{-/-}$ reticulocytes in mice still retain some ferrireductase activity.[5,15] The mRNA for *Steap2* and *Steap4*, which express ferrireductase activity,[16] are indeed consistently and significantly upregulated in bone marrow–derived macrophages and hepatocytes in the absence of *Steap3*.[17]

As α-thalassemia is a common inherited hemolytic disorder globally and can cause phenotypic alterations of erythrocytes,[18,19] we examined whether *STEAP3* mutations have synergetic effects on hematologic indices and clinical severity in α-thalassemia. Unexpectedly, we were unable to document the adverse effects of heterozygous *STEAP3* mutations on hematologic phenotypes of the α-thalassemia carriers (supplemental Figure 3). Furthermore, we identified *STEAP3* mutations in 22 subjects among the 310 HbH disease patients studied (supplemental Table 4). Also, no adverse effects were noted as a consequence of *STEAP3* mutation on the severity of HbH disease (odds ratio = 1.365, $P = .499$; supplemental Table 5).

Taken together, our findings imply that heterozygous *STEAP3* mutations are relatively common in humans and that their deleterious effect in humans remains to be confirmed. The present study, to our knowledge, represents the first comprehensive mapping of genetic alterations in the human *STEAP3* gene and shows that reduced ferrireductase activity resulting from *STEAP3* heterozygous mutations in humans fails to have a significant effect on heme synthesis.

*D.L., S.Y., and Xinhua Zhang contributed equally to this work.

Contribution: Xinhua Zhang, C.Z., R.C., Y.Z., L.L., and F.C. collected samples and clinical data; Xinhua Zhang and X.X. performed clinical classifications, diagnosis, and management of thalassemia; D.L., S.Y., P.F., L.L. Y.L., and F.C. performed laboratory and DNA analysis; Y.Y. and D.L. carried out statistical analyses; Xuelian Zhang and W.Z. did molecular diagnosis of thalassemia; N.M. and X.A. participated in the study design and interpretation of the data and edited the paper; and D.L. and X.X. designed the study, analyzed and interpreted the data, and wrote the paper.

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