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Characteristics of *TCRζ*, *ZAP-70*, and *FcεRIγ* Gene Expression in Patients with T- and NK/T-Cell Lymphoma

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Abnormal expression of key signaling molecules and defective T-cell function play a crucial role in the pathogenesis of T-cell immunodeficiency in hematological malignancies. To understand the molecular basis of T-cell signaling abnormalities and *TCRζ* chain deficiencies in T- and NK/T-cell lymphoma, the expression level of the *TCRζ*, *ZAP-70*, and *FcεRIγ* genes in peripheral blood mononuclear cells from 25 patients with T-cell lymphoma, 16 patients with NK/T-cell lymphoma (NK/T-CL), and 26 healthy individuals was determined. In addition, their relationship with disease stage and *TCRζ* 3′ untranslated region (3′UTR) splice variants was analyzed in this study. The expression level of all three genes was significantly altered with disease progression, and a decreasing trend was found in patients compared with healthy controls. *TCRζ* and *ZAP-70* were significantly positively related in all samples, and a negative relationship between *TCRζ* and *FcεRIγ* was significantly lost in NK/T-CL patients. Moreover, distinct expression patterns were defined for patient groups with different *TCRζ* 3′UTR isoforms. In conclusion, a lower expression pattern for all three genes may indicate a weaker immune status based on reduced *TCRζ* and *ZAP-70* expression without the complementary effects of *FcεRIγ*, while aberrant *TCRζ* 3′UTR splicing may contribute to T-cell receptor (TCR) signaling regulation in T cells from patients with T- and NK/T-cell lymphoma.

Introduction

Non-Hodgkin lymphomas (NHLs) are solid tumors of the immune system that represent a highly heterogeneous group of lymphoproliferative disorders. T- and NK/T-cell lymphomas are less common than B-cell lymphomas. Subtypes of this population (NHL) tend to be more clinically aggressive, and there is relatively little understanding of their molecular pathogenesis (Jaffe *et al.*, 2003; Chauchet *et al.*, 2012; Jain *et al.*, 2012; Bajor-Dattilo *et al.*, 2013; Zelenetz *et al.*, 2014). The incidence of lymphoproliferative diseases is significantly higher for individuals with congenital, acquired, or iatrogenically induced immunodeficiency (Knowles, 1999). Increasing evidence indicates that the development, maintenance, and progression of NHL are associated with disorders of the function of immune system cells, which may be due to reduced thymic output, skewed expression of the T-cell receptor (TCR) repertoire, and/or altered expression of the TCR–CD3 complex (Call *et al.*, 2002; Grulich and Vajdic, 2005).

It has been known for many years that the immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR–CD3 complex are required for initiating signaling cascades because of their recruitment and activation of multiple protein tyrosine kinases, signaling intermediates, and adapter molecules (Call and Wucherpfennig, 2005; Guy and Vignali, 2009). The *TCRζ* chain is considered to be a limiting factor for the assembly and transport of the complex to the cell surface, which is crucial for receptor signaling functions as it provides 6 of 10 immunoreceptor tyrosine-based activation motifs (ITAMs) for the complex (Call and Wucherpfennig, 2005; Li, 2008; Zha *et al.*, 2012a). *TCRζ* chain tyrosine phosphorylation is the first step in the signal transduction cascade initiated after TCR/CD3 engagement followed by phosphorylation of the cellular substrate *ZAP-70* (*TCRζ* chain-associated protein kinase 70 kDa), a cytosolic protein. The association between a lack of *ZAP-70* expression with immunodeficiency consisting of markedly reduced T-cell-mediated immunity highlights the crucial role of this tyrosine kinase in T-cell development and function (Kim *et al.*, 2006; Fischer *et al.*, 2010). The upregulation of *FcεRIγ* (Fc epsilon receptor type Iγ) expression is observed in peripheral T cells in patients with

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immunological diseases and T cells that infiltrate tumor sites (Enyedy *et al.*, 2001; Li *et al.*, 2012a). Because *FcεRIγ* is structurally and functionally homologous to the *TCRζ* chain, it was shown that it could functionally replace deficient *TCRζ* chains, facilitate TCR/CD3 complex-mediated signaling, and participate in the regulation of a variety of immune responses (Krishnan *et al.*, 2003).

The *TCRζ* mRNA is a 1472 kb spliced product of eight exons with a 492 bp coding region and a long downstream 906 bp 3′ untranslated region (3′UTR). A *TCRζ* mRNA with a deletion ranging from 672 to 1233 bp in the 3′UTR was first discovered in systemic lupus erythematosus (SLE) patients, leading to the generation of a 344 bp alternatively spliced (AS) variant lacking two critical regulatory adenosine/uridine-rich elements and a translation regulatory sequence. The AS isoform (344 bp) may have a different biological half-life or transportability, leading to the decreased expression of *TCRζ* mRNA and protein (Nambiar *et al.*, 2001; Tsuzaka *et al.*, 2002). Since it has been reported that miRNA can repress the translational of multiple protein-coding mRNAs by sequence-specific binding to the 3′UTR and may be associated with prognosis, it remains an open question whether miRNA has played a role in regulation mechanism (Gimenes-Teixeira *et al.*, 2013; Xue *et al.*, 2013). This finding indicates that the abnormal expression and dysfunction of the *TCRζ* related to the 3′UTR play a role in T-cell immune disorders. However, little is known about *TCRζ* disorders in hematological malignances, particularly in lymphocytic disease. In our previous study, we found that T cells from chronic myeloid leukemia (CML) contain different wild-type and *TCRζ* 3′UTR isoform patterns in different patients, which may be related to different upstream regulatory pathways.

Decreased or absent *TCRζ* expression in lymphocytes leads to aberrant or inefficient signaling, resulting in the partial or complete loss of immune function and its expression level and regulation in lymphocytes, which has become a critical focus for immunotherapy and immune biomarker research of malignances (Whiteside, 2004; Smedby *et al.*, 2006; Kuhns and Davis, 2007; Li, 2008; Huang *et al.*, 2012; Zha *et al.*, 2012b). Recently, data have shown that changes in the expression pattern of the *TCRζ* regulating factor, that is, the *TCRζ* 3′UTR, and the *FcεRIγ* and *ZAP-70* expression level, as well as their correlation with *TCRζ* might have distinct mechanisms in different immune-related diseases and malignancies (Smedby *et al.*, 2006; Li, 2008; Huang *et al.*, 2012; Li *et al.*, 2012a). In this study, we further analyzed the relative gene expression level of *ZAP-70* and *FcεRIγ* in a cohort of patients with T-cell NHL (T-NHL) and NK/T-cell lymphoma (NK/T-CL), where TCR signaling regulation is less well understood.

Materials and Methods

Patient selection

Forty-one lymphoma patients, including patients with T-NHL (25 cases) and NK/T-CL (16 cases) who were diagnosed according to the WHO classification, were selected for this study (26 males and 15 females, range: 12–78 years, median: 40 years), and 26 healthy individuals served as controls. The characteristics of the patients and healthy control are listed in Table 1. Details of subtype information are listed in Table 2 (all NK/T-CL cases are belonging to extranodal NK/T-cell lymphoma, nasal type). Ficoll-Paque gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs) from heparinized venous blood. The percentage of $CD3^+$ cells in PBMCs was found to be $\sim$70%. All procedures were conducted according to the guidelines of the Medical Ethics Committee of the Health Bureau of the Guangdong Province in China.

Table 1. Characteristics of the Lymphoma and Healthy Control Samples

		Gender		*Age (years)*		*Stage*			
Diagnosis	n	*Male*	*Female*	*Median*	*Range*	*I*	*II*	*III*	*IV*
T-NHL	25	13	12	42	12–78	9	2	1	13
NK/T-CL	16	13	3	39	13–74	3	2	1	10
HI	26	12	14	25	6–55				

HI, healthy individual; NK/T-CL, NK/T-cell lymphoma; T-NHL, T-cell non-Hodgkin lymphoma.

Real time polymerase chain reaction for TCRζ *3′UTR amplification*

Total RNA was isolated from the PBMC samples using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with random hexamers and reverse transcriptase using the Superscript II Kit (PowerScript Reverse; BD, San Jose, CA) according to the manufacturer's instructions. The primers used for amplification of the *TCRζ* 3′UTR and β2-microglobulin (*β2m*) gene, which was used as a control, are listed in Table 3. Real time polymerase chain reaction (RT-PCR) amplification of the *TCRζ* 3′UTR was performed as previously described (Nambiar *et al.*, 2001).

Quantitative RT-PCR

cDNA was obtained from the PBMCs of the 41 patients and 26 healthy individuals. The expression level of the *TCRζ*, *FcεRIγ*, *ZAP-70*, and *β2-microglobulin* (*β2M*) genes was determined by SYBR Green I RT-PCR. PCR was performed in a 20 μL volume with $\sim$1 μL of cDNA, 0.5 μM of each primer pair, 9 μL of 2.5×Real Master Mix (Tiangen

Table 2. Details of Subtype Information

	Subtype	*Case*
T-NHL	Anaplastic large-cell lymphoma primary systemic type (ALCL)	4
	Peripheral T-cell lymphoma, unspecified (PTCL-u)	13
	Anaplastic large-cell lymphoma primary cutaneous type (ALCL)	1
	Angioimmunoblastic T-cell lymphoma (AITL)	4
	Subcutaneous panniculitis-like T-cell lymphoma (SCPTCL)	2
	Enteropathy-type T-cell lymphoma (ETCL)	1
	Total	25

Table 3. List of Primers Used for Polymerase Chain Reaction Analysis

Primer	*Sequence (5′-3′)*	*Association number*	*Product size (bp)*
TCRζ3′-UTR	F: CAGCCAGGGGATTTCCACCACTCAAAG	NM_000734.3	906/344
TCRζ3′-UTR	R: CCCTAGTACATTGACGGGTTTTTCCTG		
TCRζ	F: GCCAGAACCAGCTCTATAAC	NM_009743.3	166
TCRζ	R: TAGGCCTCCGCCATCTTATC		
FcεRIγ	F: GAGCCTCAGCTCTGCTATATCC	NM_004106.1	172
FcεRIγ	R: TCTCGTAAGTCTCCTGGTGCC		
ZAP-70	F: GTTGACTCATCCTCAGAGACGAAT	NM_001079.3	183
ZAP-70	R: AGGTTATCGCGCTTCAGGAA		
β2M	F: TACACTGAATTCCACCCCCAC	J00105	144
β2M	R: CACTCAATCCAAATGCGGCA		

TCR, T-cell receptor; 3′UTR, 3′ untranslated region.

Biotech, Beijing, China), and 9 μL of dH_2O. The primer details are listed in Table 3 and the sequences and PCR conditions have been previously described (Huang *et al.*, 2012; Li *et al.*, 2012b; Chen *et al.*, 2013). The relative amount of the genes of interest and the *β2M* reference gene was measured in two independent assays. The $2^{(-\Delta CT)}$ method was used to analyze the results of the genes of interest relative to an internal control gene. In addition, specific amplifications of the PCR products were analyzed by melting curve analysis and electrophoresis in agarose gels.

Statistical analysis

Two independent-samples Wilcoxon tests were performed to compare the median of the differences in the mRNA expression levels between patients with T-NHL, NK/T-CL, and healthy controls. Spearman correlation and linear regression analyses were used to estimate the correlation between the mRNA levels of the different genes in different samples using the SPSS 19 statistical software. Differences with $p < 0.05$ were considered statistically significant.

Results and Discussion

Expression characteristics of the TCRζ, ZAP-70, *and* FcεRIγ *genes in T-NHL and NK/T-CL*

Previous studies have shown significant downregulation of genes related to TCR/CD3 signaling in T cells from patients with acute myeloid leukemia, CML, chronic lymphocytic leukemia (CLL), and multiple myeloma, indicating low T-cell activation in these patients. One reason for such alterations may be the direct inhibition of leukemic cells in peripheral blood (Chen *et al.*, 2011; Li *et al.*, 2011; Huang *et al.*, 2012; Zha *et al.*, 2012b). Thus, it is of interest to investigate the expression pattern of these genes in peripheral blood cells from patients with lymphomas, in which the immune suppression of T cells may be due to the tumor microenvironment rather than direct interaction with leukemic cells (Smedby *et al.*, 2006; Li, 2008).

In this study, the expression of the *TCRζ*, *ZAP-70*, and *FcεRIγ* genes in PBMCs from 41 patients with T-NHL or NK/T-CL and 26 healthy individuals was determined by real-time PCR and quantitatively assessed by comparing with the *β2M* gene, which was used as a reference. Specific amplification of the PCR products was confirmed by melting curve and agarose electrophoresis analysis. A single melting curve peak and the expected PCR products were confirmed. PCR products of all genes were randomly chosen and sequenced, and the sequencing results were confirmed by BLAST analysis for comparison with data in GenBank (data not shown). All genes were detected in every sample, and the expression levels are shown in Figure 1. Unlike the findings in leukemia patients (Chen *et al.*, 2011; Li *et al.*, 2011; Huang *et al.*, 2012; Zha *et al.*, 2012b), the expression level of the *TCRζ* gene in T-NKL (median: 0.675, $p = 0.221$) and NK/T-CL (median: 0.657, $p = 0.365$) appeared to be lower than that in healthy individuals (median: 0.731) (Fig. 1A); however, the difference was not significant. We further compared the difference in expression levels between the different T-NHL disease stages. Interestingly, a lower *TCRζ* expression level was found in stages III + IV ($n = 14$, median: 0.563) compared with stages I + II ($n = 11$, median: 0.923, $p = 0.013$) and healthy individuals ($p = 0.006$), and there was no significant difference in the *TCRζ* expression level between stages I + II and healthy individuals ($p = 0.332$) (Fig. 2A). The decrease in *TCRζ* expression may be related to disease status. A significantly lower *ZAP-70* expression level was detected in T-NHL (median: 0.242, $p = 0.008$) and NK/T-CL (median: 0.226, $p < 0.001$) compared with healthy control samples (median: 0.426) (Fig. 1B). However, there was no significant difference in *ZAP-70* expression between stages I + II (median: 0.407) and III + IV (median: 0.203, $p = 0.075$) (Fig. 2B). This finding corresponds to a study of cutaneous T-cell lymphoma in which it was found that *ZAP-70* tyrosine phosphorylation was reduced or undetectable, and the kinase weakly associated or was unassociated with the *TCRζ* chain (Fargnoli *et al.*, 1997). It is accepted that, as a downstream factor of *TCRζ*, *ZAP-70* expression is consistent with that of *TCRζ*. Moreover, *FcεRIγ* mediates signaling by associating with phosphorylated Syk protein kinase, which was found to be 100-fold more potent that *ZAP-70* and is preferentially recruited to the *FcεRIγ* receptor. In patients with immune dysfunction such as SLE or CML, *FcεRIγ* overexpression can replace a functionally deficient *TCRζ* chain and mediate signaling by associating with phosphorylated Syk protein kinase to reverse abnormal immune regulation (Enyedy *et al.*, 2001; Zha *et al.*, 2012b). Unexpectedly, the *FcεRIγ* expression level was not upregulated, but significantly downregulated in T-NHL (median: 0.559, $p = 0.013$), especially in stages I + II (median: 0.485, $p = 0.001$) compared with healthy individuals (median: 0.809) (Fig. 1C).

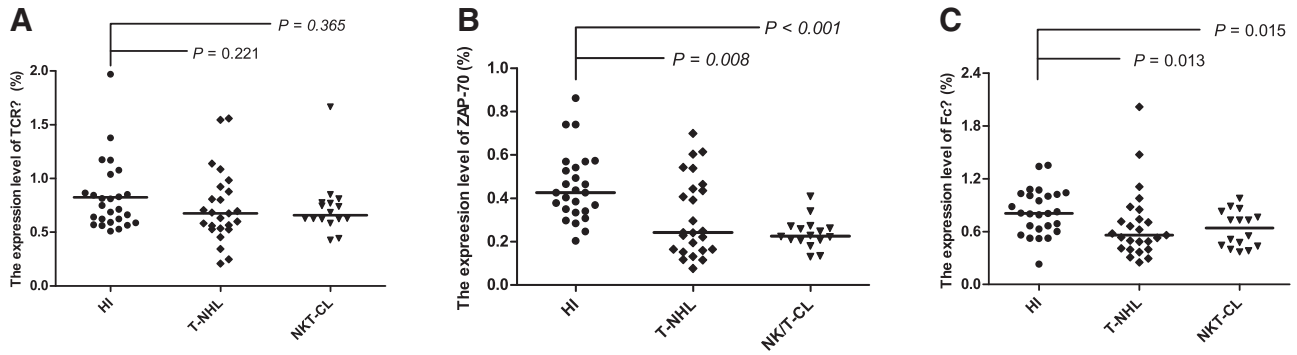

FIG. 1. The *TCRζ* **(A)**, *ZAP-70* **(B)**, and *FcεRIγ* **(C)** gene expression level in patients with T-NHL and NK/T-CL and healthy individuals. NK/T-CL, NK/T-cell lymphoma; TCR, T-cell receptor; T-NHL, T-cell non-Hodgkin lymphoma.

Interestingly, the expression level in stages III + IV was significantly higher than that in stages I + II for T-NHL (median: 0.685, $p = 0.033$, Fig. 2C). Similar trends were found for the NK/T-CL samples (median: 0.642, $p = 0.015$) in which the *FcεRIγ* expression level in stages III + IV (median: 0.730) appeared to be higher than that in stages I + II (median: 0.553, $p = 0.583$). Overall, all three genes were downregulated. Despite the growing tendency of *FcεRIγ* expression as diseases get worse, the low level in general invalidates the modification effect. These may result in a more severe impact on the T-cell immune function and a weaker immune status for patients with T- and NK/T-cell lymphoma.

The TCRζ *3′UTR isoforms and* TCRζ, ZAP-70, *and* FcεRIγ *gene expression*

Deficiencies in the *TCRζ* chain not only impair proliferative responses and the mature T-cell activation level but they also influence the TCR expression on cell membranes and the number of single-positive ($CD4^+$ or $CD8^+$) circulating T cells (Chen *et al.*, 2000). However, the mechanism responsible for the *TCRζ* absence on T cells in patients with cancer is unclear. To gain insight into the molecular mechanisms of *TCRζ* deficiency, we previously analyzed the distribution of the *TCRζ* 3′UTR isoforms, which contribute to the regulation of *TCRζ* expression in PBMCs in CML (Zha *et al.*, 2012b). In this study, we further characterized the isoform distribution of the *TCRζ* 3′UTR in samples from T-NHL and NK/T-cell patients and healthy controls.

Two *TCRζ* 3′UTR isoforms could be identified in the samples. Both PCR products were cloned, sequenced, and confirmed by comparison with the sequence found in the NCBI GenBank (data not shown). According to the characteristic distribution of the *TCRζ* 3′UTR isoforms, 26 healthy individuals and 25 T-NHL and 16 NK/T-CL cases were divided into subgroups: WT + AS −, who only express the wild-type *TCRζ* 3′UTR (906 bp); WT + AS +, who express both *TCRζ* 3′UTR isoforms (906 and 344 bp); and WT − AS +, who only express the AS *TCRζ* 3′UTR (344 bp), which is found only in the T-NHL patients (Nambiar *et al.*, 2001; Tsuzaka *et al.*, 2002). The relative expression level of the *TCRζ*, *FcεRIγ*, and *ZAP-70* genes was compared between different groups to evaluate the effects of the AS *TCRζ* 3′UTR on the expression and regulation of the *TCRζ* chain and its related genes. In general, the WT *TCRζ* 3′UTR isoform, which is thought to maintain the balance in *TCRζ* function, could be identified in all the healthy samples; however, we found three healthy cases (11.5%) containing only the WT *TCRζ* 3′UTR. Thus, it remains an open question, although there was no significant difference in the expression level of the *TCRζ* and *ZAP-70* genes between the WT + AS − and WT + AS + groups ($p = 0.355$ and $p = 0.505$, respectively) in healthy individuals.

In contrast, 24% of the T-NHL cases (6 cases) contained only the WT *TCRζ* 3′UTR isoform, and the expression levels of *TCRζ* and *ZAP-70* in the WT + AS − group are approximately twofold higher than those in the WT + AS + group ($p = 0.018$ and $p = 0.205$, respectively). This result is similar

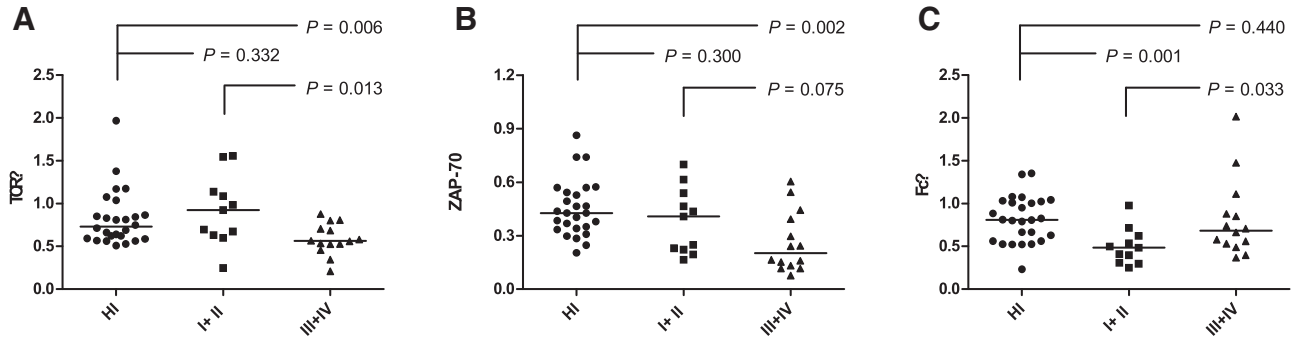

FIG. 2. The *TCRζ* **(A)**, *ZAP-70* **(B)**, and *FcεRIγ* **(C)** gene expression level in healthy individuals and T-NHL patients in stages I + II and III + IV.

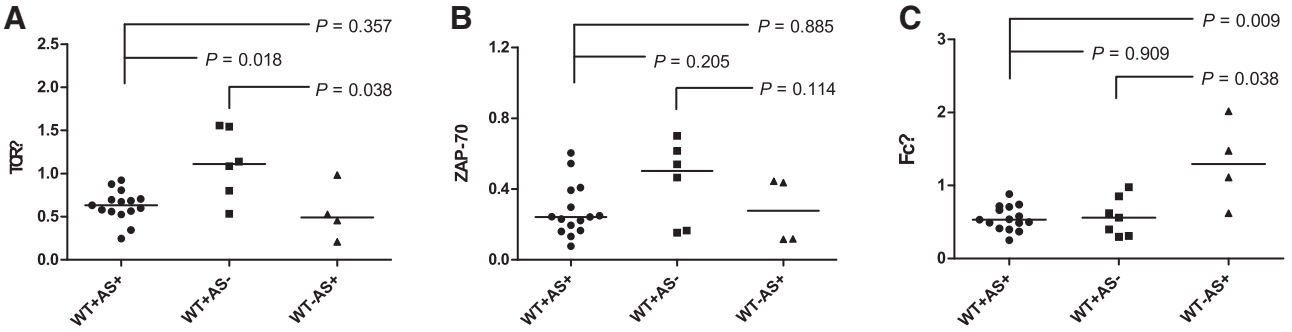

FIG. 3. The *TCRζ* **(A)**, *ZAP-70* **(B)**, and *FcεRIγ* **(C)** gene expression level in WT + AS +, WT + AS −, and WT − AS + patients with T-NHL. AS, alternatively spliced.

to the findings in CML samples. Moreover, we found that 16% (4 cases) of the T-NHL samples characteristically contained only the AS *TCRζ* 3′UTR isoform (WT − AS +), which has been detected in SLE samples (Tsuzaka *et al.*, 2002). Our data demonstrate a lower *TCRζ* and *ZAP-70* gene expression level in this group when compared with WT + AS − ($p = 0.038$ and $p = 0.114$, respectively) and WT + AS + groups ($p = 0.375$ and $p = 0.885$, respectively), while possessing a significantly higher *FcεRIγ* expression level ($p = 0.038$ and $p = 0.009$, respectively) (Fig. 3). These results may account for the feedback regulation in the immune system in certain NHL patients. As *TCRζ* mRNA stability is mainly influenced by its downstream 3′UTR, the 906 bp WT 3′UTR plays an important role in *TCRζ* transcript stability, while the AS 344 bp 3′UTR significantly influences the generation of the *TCRζ*. These results may further suggest that the AS isoforms of the *TCRζ* 3′UTR are involved in powerful gene regulation mechanisms that result in a reduced *TCRζ* expression level, partially contributing to T-cell immunodeficiency (Chowdhury *et al.*, 2005). In contrast, unlike T-NHL, CML, and SLE, only 2 cases (12.5%) were characterized in the WT + AS − group for patients with NK/T-CL lymphoma. This proportion appears to be much lower, and whether this is due to factors other than *TCRζ* 3′UTR isoform regulation or is the result of limited samples requires further investigation.

Correlation between TCRζ, ZAP-70, *and* FcεRIγ *gene expression in T- and NK/T-cell lymphoma*

To gain more insight into the mechanisms involved in abnormal TCR signal transduction, correlations between the relative *TCRζ*, *FcεRIγ*, and *ZAP-70* gene expression levels were examined. Similar to our previous study, a positive correlation between the *TCRζ* and *ZAP-70* genes was found for the healthy controls ($rs = 0.837$, $p < 0.001$) (Fig. 4A), T-cell lymphoma ($rs = 0.763$, $p < 0.001$) (Fig. 4B), and NK/T-CL patients ($rs = 0.524$, $p < 0.05$) (Fig. 4C), further supporting the correlation between the *TCRζ* and *ZAP-70* genes in T-cell activation for sound and defective cellular immunity (Kim *et al.*, 2006; Fischer *et al.*, 2010). *FcεRIγ*

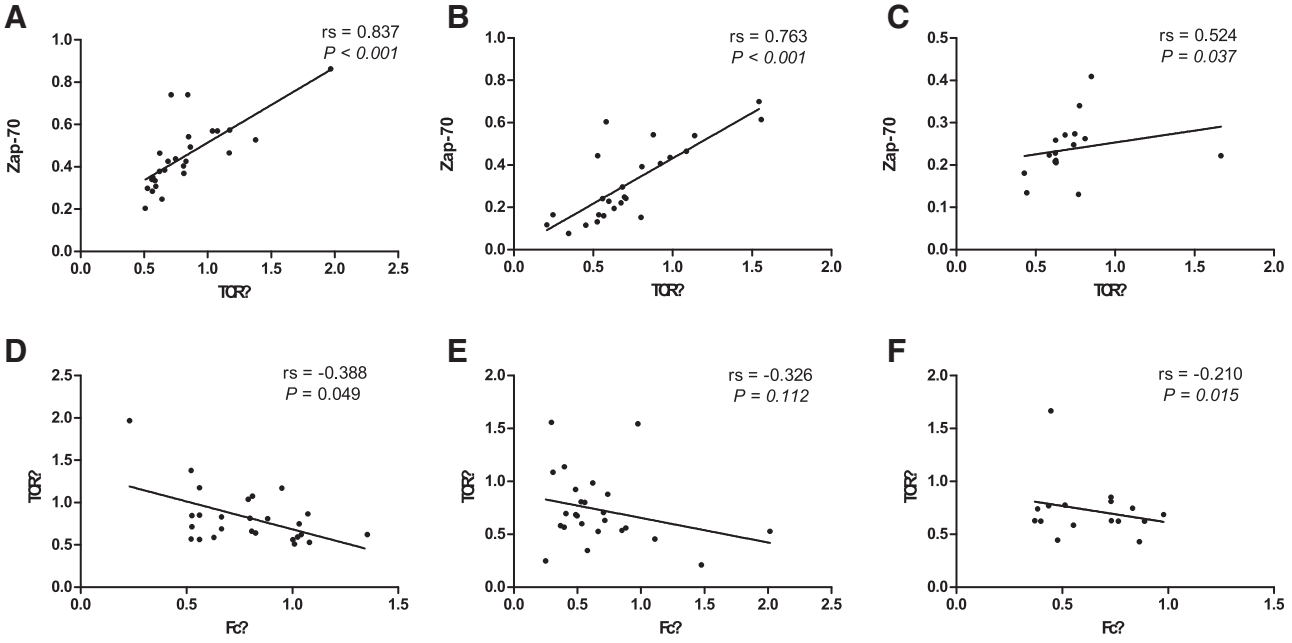

FIG. 4. Correlation analysis between the *TCRζ* and *ZAP-70* gene expression level in HI **(A)**, T-NHL **(B)**, and NK/T-CL **(C)** patients and the *TCRζ* and *FcεRIγ* gene expression level in HI **(D)**, T-NHL **(E)**, and NK/T-CL patients **(F)**. HI, healthy individual.

functions like a candidate for replacing *TCRζ* through its association with *Syk*, which is regarded to be 100-fold more potent than *ZAP-70* and preferentially recruited to the *FcεRIγ* receptor (Krishnan *et al.*, 2003). A negative correlation was observed between the *TCRζ* and *FcεRIγ* expression levels, and it was statistically significant for the healthy control ($rs = -0.388$, $p < 0.05$) (Fig. 4D) and NK/T-CL samples ($rs = -0.210$, $p = 0.015$) (Fig. 4F). Although a tendency toward a negative correlation was also detected for the T-NHL samples ($rs = -0.326$, $p = 0.112$) (Fig. 4E), there was no significance in these samples. These results are similar to that of our previous study in CLL (Huang *et al.*, 2012), in which we suggested that the *FcεRIγ* expression level is incapable of substituting for *TCRζ* deficiency or contributing to TCR signal transduction in a manner similar to conserved functional ITAMs. Because there is abnormal immune regulation and no immunodeficiency in SLE (Enyedy *et al.*, 2001), this phenomenon may indicate an even worse immune statue without regulation even in patients with immunodeficiency.

In conclusion, we characterized for the first time the *TCRζ*, *ZAP-70*, and *FcεRIγ* gene expression pattern in patients with T- and NK/T-cell lymphoma and showed that the decreasing trend in the expression level of all three genes was significantly associated with disease progression, which may indicate an even weaker immune status according to reduced *TCRζ* and *ZAP-70* expression without complementary *FcεRIγ* effects. Moreover, distinct *TCRζ* 3′UTR isoform expression patterns in patients suggest that aberrant *TCRζ* 3′splicing may contribute to TCR signaling regulation in peripheral blood T cells from patients with T- and NK/T-cell lymphoma.

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Authors' Contribution

Y.Q.L. contributed to the concept development and study design. Z.W.L. and L.L.Z. performed real-time PCR. S.H.C. and L.J.Y. prepared PBMCs. Z.F.H. and X.W. prepared RNA and cDNA. C.Y.W., X.D.L., and H.T. were responsible for clinical diagnoses and performed clinical data acquisition. Y.Q.L. and Z.W.L. coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript.

Disclosure Statement

The authors declare that they have no competing interests.

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