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Implicated role of liposarcoma related fusion oncoprotein TLS-CHOP in the dysregulation of arginine-specific methylation through PRMT1

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Abstract: Chromosomal translocation product, TLS-CHOP (Translocated in liposarcoma-CCAAT/enhancer binding protein homologous protein, also named as FUS-DDIT3), has been thought to be a primary cause of myxoid liposarcoma, but the precise molecular function of TLS-CHOP for oncogenesis still remains to be elucidated. Previously we demonstrated that TLS/FUS interacts with protein arginine methyltransferase 1 (PRMT1), and carboxyl-terminal region of TLS is dimethylated by PRMT1. However, it has been uncovered whether TLS-CHOP function is regulated by PRMT1, and is methylated. Here we indicate that TLS-CHOP is not associated with PRMT1 and less methylated even though TLS-CHOP still possesses several potential arginine methylation sites of TLS. Moreover, we established a stable cell line expressing TLS-CHOP as a model system for studying the molecular function of TLS-CHOP. The TLS-CHOP expressing 293T cells exhibited slight growth retardation and decreased level of integrin α5β1 protein, a fibronectin receptor. It would be possible that the expression of oncoprotein TLS-CHOP might dysregulate arginine-specific methylation elicited via PRMT1 interacting with methylated TLS.

Keywords: TLS-CHOP, FUS-DDIT3, PRMT1, Arginine Methylation, Liposarcoma

1. Introduction

TLS (Translocated in LipoSarcoma; also termed FUS, FUsed in Sarcoma)-CHOP (CCAAT/enhancer-binding protein Homologous Protein; also named DDIT3, DNA Dameged-Inducible Transcript 3) was discovered as an oncogenic fusion gene product by chromosomal translocation t(12;16)(q13;p11) in myxoid liposarcoma patients [1] [2]. Since TLS-CHOP fusion transcripts are detected in over 90% of myxoid liposarcoma patients, TLS-CHOP is used as a diagnostic marker for myxoid liposarcoma [3]. Oncogenic property of TLS-CHOP has been indicated by transformation assay using mouse NIH-3T3 cells, ST-13 cells, and transgenic animal models [4-6]. TLS-CHOP is a fusion protein of amino-terminal (1-266 residues) of TLS/FUS and full length of CHOP/DDIT3 by reciprocal translocation [1]. TLS is a multifunctional protein, which regulates transcription, pre-mRNA splicing, DNA repair and homologous recombination [7-12]. Recently several sites of missense mutation have been identified in the carboxyl-terminal region of TLS in familial amyotrophic lateral sclerosis (ALS) [13-15]. Meanwhile, the wild-type CHOP protein product possesses a leucine zipper domain and acts as a stress-responsive transcription factor [16]. Therefore, it is

believed that TLS-CHOP is an aberrant transcription factor or mutated pre-mRNA splicing factor, and promotes the oncogenesis [17].

The TET family of proteins is composed of TLS, EWS (Ewing sarcoma), and TAF15 (TBP-associated factor 15) and share structural features [18]. Although chromosomal translocations of TET family proteins (e.g. EWS-FLI1, TAF15-NOR1) are detected in a part of sarcoma and leukemia [19], detailed physiological functions of TET family proteins and their sarcoma-specific fusion protein products remain unclear.

We have demonstrated that TLS is associated with protein arginine methyltransferase 1 (PRMT1), a major protein methyltransferase for asymmetric dimethylarginine (ADMA) modification, and dimethylated at several arginine residues by PRMT1 [8]. A limited number of PRMT1 interacting proteins have been described to affect PRMT1 activity such as CAF1 and its related protein BTG1 [20, 21], and little is known how PRMT1 activity is regulated. Several evidences suggest that aberrant PRMT activity possibly plays an important role in oncogenesis [22-24]. Altered status of ADMA-modifications regulated by PRMT1 could be a potential cause of myxoid liposarcoma. TLS-CHOP fusion protein expression in liposarcoma may dysregulate post-translational modification of arginine residues by PRMT1 with methylated TLS.

In this study, we show that TLS-CHOP has no interaction with PRMT1, and is less methylated in 293T cells. We also established the 293T cells expressing TLS-CHOP stably, which is a beneficial tool to investigate the oncogenic pathways of myxoid liposarcoma. Over-expression of TLS-CHOP reduced cell adhesion due to down-regulation of integrin $\alpha 5\beta 1$, a fibronectin receptor [25]. Our findings suggest that TLS-CHOP may dysregulate arginine-specific methylation elicited via PRMT1 interacting with TLS. The dysregulation of methylation by TLS-CHOP could be one of potential causes for myxoid liposarcoma.

2. Materials and Methods

2.1. Plasmid Construction

The TLS-CHOP/FUS-DDIT3 cDNA plasmid, kindly gifted from Dr. D. Ron (New York University School of Medicine), was subcloned into the pIRESpuro 3 (BD Biosciences) and pcDNA3.1(-) (Invitrogen) vectors. The FLAG-tagged human TLS expression vector was described previously [8].

2.2. Cell culture and Establishment of Stable Cell Lines

Human embryonic kidney 293T cells were maintained in RPMI1640 (Nakalai tesque) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc.). Trasnsfection was performed using Lipofectamin 2000 (Invitrogen) according to the manufacture's instruction. To generate stable cell lines, pIRESpuro3 empty vector or pIRESpuro3-TLS-CHOP vector was transfected into 293T cells. After 2 days later, the transfected cells were passaged into flesh growth medium containing puromycin (10 μg/ml), and maintained selection pressure by keeping puromycin in the growth medium for 8 days.

2.3. Western Blotting and Immunoprecipitation

Whole cell extracts were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA). The cell extracts were incubated with FLAG-M2 agarose beads (Sigma-Aldrich) for 1 hour and washed with RIPA buffer three times. The bound proteins were eluted with SDS-PAGE sample buffer. Western blotting was performed with the following antibodies; TLS/FUS (611385, BD Biosciences), CHOP/DDIT3/GADD153 (SC-793, Santa Cruz Biotechnology, Inc.), β-actin (A-5441, Sigma-Aldrich), PRMT1 (#2449, Cell Signaling Technology), FLAG (F3165, Sigma-Aldrich), anti-dimethyl-arginine antibody, asymmetric (ASYM24) (07-414, Millipore), integrin $\alpha 5$, $\beta 1$and $\beta 3$ (#4705, #4706 and #4702, Cell Signaling Technology).

3. Results

3.1. TLS-CHOP has no Interaction with PRMT1 and is Less Methylated

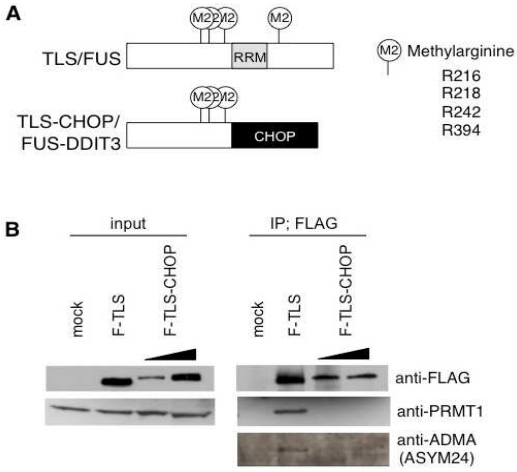

Figure 1. *TLS-CHOP/FUS-DDIT3 has no interaction with PRMT1 and is less methylated. (A) Structure of TLS/FUS and TLS-CHOP/FUS-DDIT3 proteins. The circles indicate arginine dimethylation sites. Four arginine residues (R216, R218, R242, and R394) are consistently dimethylated as shown in [8]. TLS-CHOP still possesses the possible methylation resides of arginine and lacks the RNA recognition motif (RRM) due to translocation. (B) TLS-CHOP has no interaction with PRMT1 and is less dimethylated. The whole cell extracts (20 μg) prepared from transiently expressed FLAG-tagged TLS or FLAG-tagged TLS-CHOP in 293T cells was immunoprecipitated with anti-FLAG antibody. Sixty μg of TLS-CHOP transfected cell extracts were also used for the immunoprecipitation to adjust the expression levels between FLAG-tagged TLS and FLAG-tagged TLS-CHOP. The precipitated PRMT1 bound with FLAG-tagged proteins was detected by Western blotting with anti-PRMT1 antibody. Arginine-methylation status of the precipitated TLS and TLS-CHOP proteins was monitored with anti-dimethyl arginine, asymmetric, antibody (ASYM24). Note that TLS-CHOP does not associate with PRMT1 and is less methylated.*

We demonstrated that TLS is associated with PRMT1 and is methylated at four arginine residues (R216, R218, R242 and R394; Fig. 1A) [8]. Then, we inquired whether TLS-CHOP, an oncogenic fusion protein of TLS and CHOP by translocation, could associate with PRMT1 and be methylated by PRMT1 as TLS-CHOP lacks methylation site of R394 (Fig. 1A). To examine it, FLAG-tagged TLS or TLS-CHOP was transiently overexpressed in human embryonic kidney 293T cells, and co-immunoprecipitation assays were performed with a mouse anti-FLAG antibody. Somehow FLAG-tagged TLS-CHOP was not overexpressed well although the transfected cells appeared to be wild-type cells. Consistent with our previous data [8], endogenous PRMT1 was co-immunoprecipitated with FLAG-tagged TLS in 293T cells (Fig. 1B, middle panel). Contrarily, PRMT1 was not co-immunoprecipitated with FLAG-tagged TLS-CHOP (Fig. 1B, middle panel). Since PRMT1 is a major type I protein arginine methyltransferase to generate asymmetrically dimethylated arginines (ADMA), we carried out Western blotting with anti-ADMA antibody (ASYM24) to detect proteins containing asymmetrically dimethylated arginines. Immunoprecipitated FLAG-tagged TLS was clearly asymmetrically dimethylated, while immunoprecipitated FLAG-tagged TLS-CHOP was less dimethylated (Fig. 1B, bottom panel). This result suggests TLS-CHOP has no association with PRMT1, and is less dimethylated in 293T cells even though TLS-CHOP has putative arginine methylation sites in its protein sequence (Fig. 1A, B)

3.2. Establishment of TLS-CHOP Expressing Cells as a Model System for Studying the Molecular Function of TLS-CHOP.

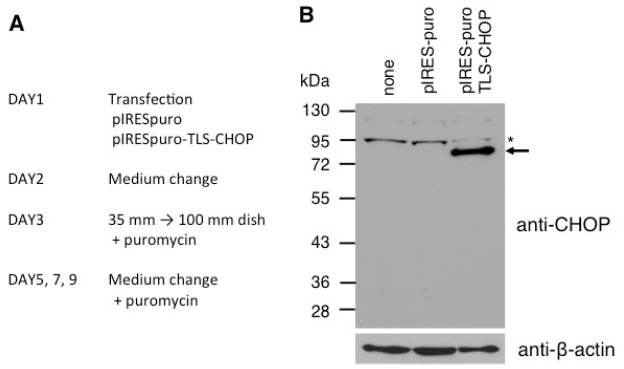

Figure 2. *Establishment of TLS-CHOP expressing 293T cells. (A) Scheme for generating the stable TLS-CHOP expressing cells. pIRESpuro3 empty vector or pIRESpuro3-TLS-CHOP vector was transfected into 293T cells. After drug selection, viable cells were obtained. (B) Confirmation of TLS-CHOP ectopic expression. The ectopic expression of TLS-CHOP in 293T cells was detected by Western blotting with anti-CHOP/DDIT3 antibody. Arrow indicates the TLS-CHOP fusion protein, and star indicates nonspecific signals. β-actin was used as a loading control.*

To investigate the molecular function of TLS-CHOP for oncogenesis, we executed a plan to generate a human cell lines expressing TLS-CHOP stably. After working with MCF-7, HeLa, A549, and 293T cells, we have obtained puromycin-resistant cells only from 293T cells, which were introduced with TLS-CHOP-IRES-puromycin resistant cassette. The transfected 293T cells were incubated in culture medium containing puromycin. The ectopic expression of TLS-CHOP in viable 293T cells was confirmed by Western blotting with anti-CHOP-antibody (Fig. 2B). As expected, endogenous CHOP expression (predicted molecular weight; 30 kDa) was not detected in any of cells (Fig. 2). Stable cell lines expressing TLS-CHOP has never been created from MCF-7, HeLa, and A549 cells, suggesting that TLS-CHOP might serve for cell linage selective oncogenic transformation.

3.3. The TLS-CHOP Expressing 293T Cells Exhibit Changes in Adherence and Growth

We noticed that the TLS-CHOP expressing 293T cells exhibited slightly smaller and rounder morphology compared with the control cells (Fig. 3A). The stable TLS-CHOP cell line required longer time to fully attach on plates, that is, parental and empty vector-introduced 293T cells completed adhesion on bottom of the plates, whereas, the stable cell line attached partially 24 hours after seeding the cells (Fig. 3A). We also counted cell numbers to check cellular growth, and found that the stable cells expressing TLS-CHOP grow slightly slow (Fig. 3B)

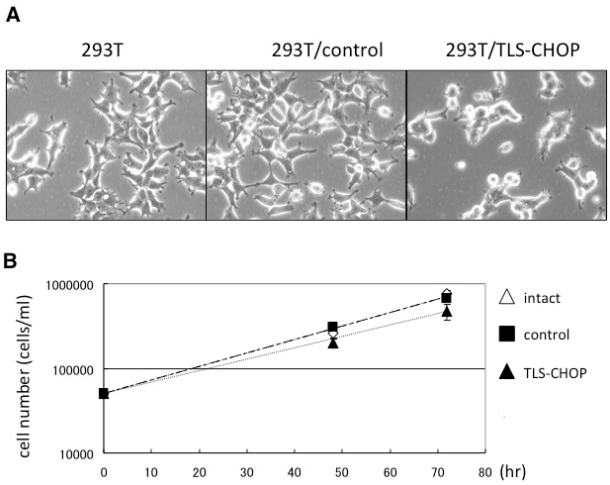

Figure 3. *Expression of TLS-CHOP in 293T cells alters cell morphology and adhesion. (A) Phase-contrast images of untransfected (left), empty-vector transfected (middle), and TLS-CHOP expressing 293T cells (right). The phase-contrast images (x100) were taken at the 24 hrs after seeding. The TLS-CHOP expressing 293T cells exhibited slightly smaller and rounder morphology, and weakly attached to the plate. (B) Expression of TLS-CHOP inhibits cellular growth of 293T cells. The number of TLS-CHOP expressing 293T cells was counted at 48 and 72 hours. The stable cell line displayed slight growth retardation.*

3.4. The Ectopic Expression of TLS-CHOP in 293T cells decrease the level of cell adhesion-related protein integrin α5 and β1

As the stable cell line reduced cell adhesion, we investigated the cell adhesion-related proteins regulated by TLS-CHOP by using series of anti-integrin antibodies. The

expression of integrin α5 and β1, a major receptor for fibronectin, was decreased substantially in the stable TLS-CHOP 293T cell line (Fig. 4). On the other hand, integrin β3 expression had no difference between the control and the TLS-CHOP expressing cells. The expression of integrin α5 and β1 mRNA was not changed (data not shown), suggesting TLS-CHOP should regulate integrin α5 and β1 expression at the post-transcriptional level.

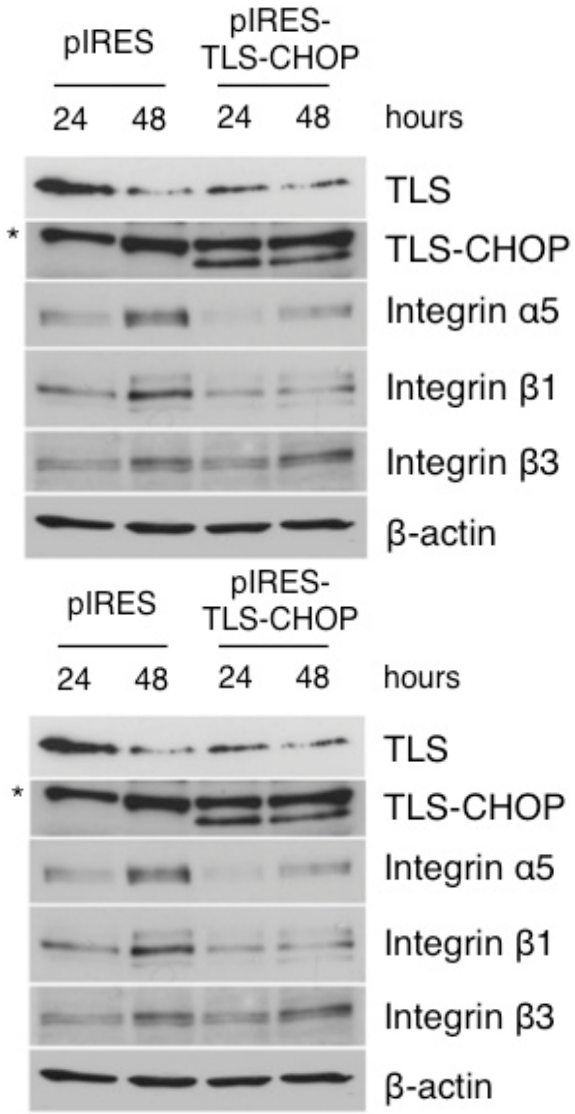

Figure 4. *TLS-CHOP expressing 293T cells decrease the level of cell adhesion-related protein integrin α5 and β1. The whole cell extracts were prepared at 24 and 48 hrs after seeding, and twenty μg of cell extracts was used for Western blotting. The empty vector introduced 293T cells were used as a control. β-actin was used as a loading control. Star indicates nonspecific signals. The expression of integrin α5 and β1 was decreased in TLS-CHOP expressing 293T cells.*

4. Discussion

TLS-CHOP/FUS-DDIT3 was originally identified as an oncogenic fusion gene by chromosomal translocation in myxoid liposarcoma patients. TLS-CHOP expression has been studied only in a few cell lines [26-28], and the molecular function of TLS-CHOP is unclear. We previously indicated that TLS is associated with PRMT1 and arginine-methylated (Fig. 1B) [8]. It has shown that PRMT1 regulates cell proliferation in human cancer cell lines [29], and needs associated proteins such as TIS21, BTG1 and hCAF1 for its enzymatic activity [20-21]. Here we demonstrated that TLS-CHOP has no interaction with PRMT1 and is less methylated (Fig. 1B). It is interesting to note that alteration of protein binding specificity by chimeric protein fusion was reported in the case of TLS, TLS-CHOP and splicing factor YB-1 [17]. Our results suggest TLS-CHOP will not act as a normal modulator for PRMT1 due to less binding with it. It is conceivable that methylated TLS and its binding complex act as a modulator of PRMT1, and TLS-CHOP may disrupt the function of TLS and PRMT1 such as mRNA splicing, arginine-specific methylation.

Stable transfection of TLS-CHOP into NIH/3T3 cells leaded to morphological changes [4]. Here we established the TLS-CHOP stably expressing cell lines based on 293T cells (Fig. 2), and demonstrated that these cells exhibit slight growth retardation and down-regulate integrin α5β1 protein expression (Fig. 3B and 4). Our results suggest that TLS-CHOP plays a role in regulation of integrin mediated-signals for cell survival and metastasis for tumor cells.

Altered status of ADMA-modification could be a potential cause of myxoid liposarcoma. Several evidences suggest that aberrant PRMT activity possibly plays an important role in oncogenesis [22-24]. TLS-CHOP expression may interrupt post-translational modification of arginine residues by PRMT1 with TLS. Another possibility of the liposarcoma formation induced with TLS-CHOP might be attributed to dysregulation of expression of the CHOP target genes. Further analysis of down-stream pathway(s) and/or target(s) in arginine methylation will be helpful to develop the therapeutics for myxoid liposarcoma.

5. Conclusions

We demonstrate that the myxoid liposarcoma-associated fusion protein TLS-CHOP/FUS-DDIT3 does not interact with PRMT1 and is less methylated. The ectopic expression of TLS-CHOP reduces proliferation of 293T cells, and decreases the levels of integrin α5 and β1 proteins. Our results implicate that TLS-CHOP may dysregulate arginine-specific methylation elicited via PRMT1 interacting with TLS. Further analysis of down-stream pathway(s) and/or target(s) in arginine methylation will be helpful to understand the pathogenesis of myxoid liposarcoma.

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