Unfolded protein response suppresses CEBPA by induction of calreticulin in acute myeloid leukaemia

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Abstract

The unfolded protein response (UPR) is triggered by the accumulation of misfolded proteins within the endoplasmic reticulum (ER). The role of the UPR during leukemogenesis is unknown so far. Here, we studied the induction of mediators of the UPR in leukaemic cells of AML patients. Increased expression of the spliced variant of the X-box binding protein 1 (XBP1s) was detected in 17.4% (16 of 92) of AML patients. Consistent with activated UPR, this group also had increased expression of ER-resident chaperones such as the 78 kD glucose-regulated protein (GRP78) and of calreticulin. Conditional expression of calreticulin in leukaemic U937 cells was found to increase calreticulin binding to the CEBPA mRNA thereby efficiently blocking translation of the myeloid key transcription factor CEBPA and ultimately affecting myeloid differentiation. Consequently, leukaemic cells from AML patients with activated UPR and thus increased calreticulin levels showed in fact suppressed CEBPA protein expression. We identified two functional ER stress response elements (ERSE) in the calreticulin promoter. The presence of NFY and ATF6, as well as an intact binding site for YY1 within these ERSE motifs were essential for mediating sensitivity to ER stress and activation of calreticulin. Thus, we propose a model of the UPR being activated in a considerable subset of AML patients through induction of calreticulin along the ATF6 pathway, thereby ultimately suppressing CEBPA translation and contributing to the block in myeloid differentiation.

Keywords: UPR • AML • Calreticulin • CEBPA • ATF6

Introduction

One of the transcription factors crucial for normal myeloid cell development is the CCAAT/enhancer binding protein alpha (CEBPA) [1–5]. Targeted disruption of CEBPA results in a block of granulocyte maturation [6], whereas expression of CEBPA in precursor cells is sufficient to trigger granulocytic differentiation [7]. In AML patients, deregulation of CEBPA function is a common event comprising genomic mutations [8–10], transcriptional suppression [11] and functional inactivation [12, 13]. Furthermore, CEBPA protein is suppressed in chronic myeloid leukaemia in blast crisis by the poly(rC)-binding protein hnRNP E2 [14]. The RNA-binding protein calreticulin can be specifically induced in core binding factor AML [15, 16].

The unfolded protein response (UPR) is triggered by the accumulation of misfolded proteins in the endoplasmic reticulum (ER). It reduces the protein load entering the ER by lowering the global protein synthesis and by increasing the capacity to handle misfolded proteins through activation of ER chaperone molecules [17, 18]. If homeostasis cannot be achieved cell death is triggered [19, 20]. Three pathways of ER-stress transduction have been identified comprising the inositol-requiring protein-1 (IRE1), the activating transcription factor 6 (ATF6) and the protein kinase RNA-like ER kinase (PERK) [21]. In the case of IRE1, ER-stress induces the cleavage of the X-box binding protein 1 (XBP1) mRNA encoding a novel potent transcriptional activator of UPR target genes [22–25]. Other cellular targets of the UPR are calreticulin, CHOP and GRP78.

In this report, we found that the UPR is activated in 17.4% of AML patients as determined by the induction of the XBP1 spliced variant and increased expression of GRP78 and calreticulin. At the molecular level, we found that the activation of the calreticulin promoter following ER-stress was mediated by two copies of an ER-stress response element (ERSE) [27–30]. We identified YY1, the nuclear
transcription factor Y (NF-Y) and the nuclear form of the activating transcription factor 6 (ATF6n) as ERSE-binding transcription factors. These results suggest a role of the UPR during leukemogenesis including early induction of calreticulin, thereby suppressing CEBPA translation and inhibiting myeloid differentiation.

Materials and methods

Patients

Malignant cells were collected at the time of diagnosis before initiation of treatment. They were prepared from Ficoll-separated mononucleated cells of bone marrow aspirates from 92 consecutive AML patients seen at the Department of Medical Oncology, University Hospital, Bern, Switzerland between 2002 and 2004. Informed consent from all patients was obtained according to the Declaration of Helsinki, and the studies were approved by decisions of the local ethics committee of Bern, Switzerland. Conventional karyotype analysis of at least 20 metaphases was performed for each patient.

Cell lines and reagents

Human lung cancer H1299 and myeloid leukaemic K562 and U937 cells were grown in RPMI 1640 (SIGMA, Buchs, Switzerland) supplemented with 10% fetal bovine serum (FBS) (PAA, Pasching, Austria). For induction of ER-stress, cells were incubated with Thapsigargin (300 nM, final concentration), Calcimycin A23187 (7 μM, final concentration) or Tunicamycin (10 μg/ml), all obtained from SIGMA (Buchs, Switzerland) and dissolved in DMSO.

RT-PCR and assay to detect the spliced variant of XBP1 mRNA (XBP1s)

RT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Taqman PCR Mix (Roche, Branchburg, NJ, USA). For GRP78, calreticulin, ATF4 and ABL, assay-on-demand gene expression probes (Applied Biosystems) were applied. The relative quantization was indicated by Ct values, determined based on duplicate reactions for each target and internal control gene [31]. To assess the induction of XBP1s by PCR, the following primers were used: (s) 5’-GGGAATGAAGTGAGGCCAG-3’ and (as) 5’-CAATACCGCCA-GAATCCATG-3’ corresponding to nucleotides 412–431 and 834–853 of the 

Complementary oligonucleotides were labeled using γ-32P-ATP (GE Healthcare Ltd Amersham, Buckinghamshire, UK). EMSA was performed as previously described [8, 15]. For super-shift analyses, 4 μg of antibody (ATF6, YY1; all from Santa Cruz Biotech, Santa Cruz, CA, USA) were incubated with nuclear extracts. Sequences of the oligonucleotides are given in Table S1 as supplemental material.

Chromatin immunoprecipitation (ChIP) assay

The ChIP-IT kit (Active Motif, Rixensart, Belgium) was used according to the manufacturer’s instructions. Isolated chromatin of K562 cells was incubated with antibodies against NF-Y-A, YY1 or ATF6, all from Santa Cruz Biotechnology. PCR primers for calreticulin were: (s) 5’-CCTGGCCTGA-GATCCATG-3’ and (as): 5’-G6TGTCCTCG-3’; and (as): 5’-AGGCTGCGCCTCGGAACG-3’; intron 7 (s): 5’-GTTGACA-GAATTGAGATCG-3’ and intron 7 (as): 5’-ACCTGGGTAGCA-GAGCAAG-3’.

Western blot analyses

The following antibodies were used against calreticulin (sc-6467), GRP78 (sc-13968), YY1 (sc-7341X), XBP1 (sc-7160X), CEBPA (sc-61) and SP1

Constructs

The human HA-tagged ATF6 plasmid was provided by Ron Prywes [32]. The plasmid for the nuclear form of human ATF6n was a gift from Katzutoshi Mori [23]. The plasmid for YY1 was from Yang Shi [33]. A fragment of human calreticulin promoter spanning nucleotide –1175 to +72 was amplified from DNA of peripheral blood lymphocytes from a healthy volunteer and cloned into the KpnI-Xhol sites of the pGL3 luciferase vector.

Transfection conditions and reporter gene assays

H1299 cells were transfected with 80 ng of reporter plasmid and 100 ng of pCMV-ATF6-373 or pCMV empty vector together with 0.5 ng of the CMV-Renilla reference plasmid using Lipofectamine 2000® (Invitrogen, Carlsbad, CA, USA). Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). In Hela cells, 200 ng of reporter plasmid were transfected together with 0.5 ng of the CMV-Renilla reference plasmid. Eight hours after transfection, cells were treated with thapsigargin (300 nM), calcimycin A23187 (7 μM) or tunicamycin (10 μg/ml). Luciferase activities were measured after 16 hrs. Fold induction was defined as the ratio of induced levels of activity compared to the level of activity in cells treated with DMSO alone. Each transfection experiment was repeated at least three times.

Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotides were labeled using γ-32P-ATP (GE Healthcare Ltd Amersham, Buckinghamshire, UK). EMSA was performed as previously described [8, 15]. For super-shift analyses, 4 μg of antibody (ATF6, YY1; all from Santa Cruz Biotech, Santa Cruz, CA, USA) were incubated with nuclear extracts. Sequences of the oligonucleotides are given in Table S1 as supplemental material.
Types. Using PCR primers spanning the critical 26 nucleotides of mononucleated bone marrow samples of 92 patients of all sub-UPR in AML patients at diagnosis, we screened mRNA from molecular weight of 54 kD. In order to assess the activation of the frame shift and thus to a novel potent transcription factor at a repeats. The UV cross-link assay was previously described [15, 34].

5'-UAACCAGCCGCCGCCGCCGCCGCCGCCGCCCC-3' containing CCG repeats. The UV cross-link assay was previously described [15, 34].

Results

The XBP1 spliced variant (XBP1s) is expressed in a subset of AML patients

The unfolded protein response (UPR) involves the cleavage of the XBP1 mRNA generating a spliced form (XBP1s) [23]. Activated IRE1 excises 26 nucleotides from the XBP1 mRNA leading to a frame shift and thus to a novel potent transcription factor at a molecular weight of 54 kD. In order to assess the activation of the UPR in AML patients at diagnosis, we screened mRNA from mononucleated bone marrow samples of 92 patients of all sub-types. Using PCR primers spanning the critical 26 nucleotides of the XBP1 cDNA, we determined the expression of the spliced and unspliced forms of XBP1 mRNA (Fig. 1A). The threshold of the assay was determined by competitive PCR using constant amounts of XBP1 wild-type plasmid combined with increasing amounts of the spliced form of XBP1 (Fig. S1). The ratio observed with 30 ng of wild-type XBP1 and 20 ng of the spliced form of XBP1 was selected to separate AML patients with induced versus uninduced XBP1s mRNA. We found that the XBP1s mRNA variant was present (S+) in 16 of 92 patients (17.4%), whereas it was not detectable (S-) in mononuclear cells from bone marrow samples of 10 healthy individuals. Characteristics of S+ and S- patients are summarized in a supplemental Table S2. Bone marrow was available at remission from three patients with activated UPR in leukemic cells at diagnosis. Consistently, induced XBP1s mRNA was undetectable at remission. In addition, selected CD34+ cells obtained at remission during stem cell collection in two patients, who had activated UPR at diagnosis, showed no evidence of XBP1s induction.

Relative expression levels of GRP78 and calreticulin are specifically induced in AML patients with activated UPR (S+)

To confirm activation of the UPR in AML patients with induced XBP1s, we determined mRNA expression levels of UPR target genes such as GRP78 and calreticulin. The mean ΔCt-value of patients diagnosed with FAB-subtype M0 served as reference (1-fold). In the group of S+ patients, median expression levels were increased for calreticulin (2.5-fold) and GRP78 (2.3-fold) as compared to S- patients (Figs. 1B and C). The increased expression observed for these UPR target genes in S+ patients supports the concept that the UPR is activated in these AML patients. Remarkably, we found no difference in CEBPA mRNA levels between S(+) and S(-) patients (Fig. S2).

Induction of ER-stress transcriptionally activates calreticulin expression

Thapsigargin triggers the UPR by blocking the endoplasmatic Ca-ATPase leading to depletion of ER calcium stores. We treated adherent Hela and leukaemic K562 cells with 300 nM thapsigargin. We detected splicing of the XBP1 mRNA after 2 hr of thapsigargin treatment in both cell lines (Fig. 2A). Thus, XBP1 splicing is an early event during the UPR.

Similarly, increased calreticulin mRNA expression was detectable after 2 hrs of UPR inducing treatment in Hela cells (Fig. 2B). Results were similar for three different UPR inducing compounds (A23187, thapsigargin and tunicamycin). After 16 hrs, the mean increase of calreticulin mRNA was 2.5-fold (SD ± 1.1) in K562 cells (Fig. 2C) and 4.1-fold (SD ± 1.1) in Hela cells (Fig. 2B). The induction of calreticulin protein in K562 cells following UPR activation is depicted in Fig. 2D. Expression of additional UPR chaperons such as GRP78, CHOP and the nuclear form of ATF6 was similarly induced. We thus conclude that thapsigargin treatment induces mediators of the UPR in myeloid leukemic cells.

Two elements mediate transcriptional activation of the calreticulin promoter following activation of the UPR

Our results previously mentioned suggest that induction of the UPR transcriptionally activates calreticulin. In order to define the elements mediating this activation, myeloid U937 cells were transfected with a series of deletion constructs of the calreticulin promoter cloned into the pGL3b-luciferase plasmid, and subsequently treated with 300 nM thapsigargin for 16 hrs, starting 8 hrs after transfection (Fig. 3A). We observed a two-step activation of the calreticulin promoter: A 2-fold increase of activity was measured for the −298 nt construct, and an additional increase was observed with the −205 nt construct, whereas larger constructs failed to mediate additional activity.

This region of the calreticulin promoter harbours two putative ERSE designated as ERSE1 and ERSE2 (Fig. 3B). ERSE1 has an inverse orientation of the ERSE consensus sequence CCAAT-N9-CCACG, whereas ERSE2 differs in the last 3' two nucleotides from the consensus sequence. We transfected U937 cells with specific mutation constructs of ERSE1, ERSE2 or both, again with subsequent

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treatment with 300 nM thapsigargin. For ERSE1, the mutations were cloned into the –298 nt construct (Fig. 3C), and for ERSE2 into the –205 nt construct (Fig. 3D). We found that activation following ER-stress was completely abolished when the CAAT motifs of both ERSE1 and ERSE2 were mutated (construct M4 in Fig. 3C). Specific mutation of a putative ATF6 binding site in ERSE1 (M2), as well as mutation of the CAAT motif (M1) reduced the activation potential to that seen with the wild-type ERSE2 alone (–205 nt construct). Mutation of the putative YY1-binding site in the ERSE1 motif (M3) had no effect on activation.

The ERSE2 motif encodes no obvious ATF6 binding site. Mutation of the CAAT motif (M5) or the putative binding site of YY1 (M6) completely abolished the activation potential of the ERSE2 motif (Fig. 3D). Reduction of the 9 nucleotides spacer sequence between the CAAT motif and the YY1-binding site to 7 nucleotides (M8) also inactivated the promoter construct following induction of ER-stress. In contrast, exchanges of two nucleotides per se within the spacer sequence (M7) had no effect. In summary, both ERSE sequences appear to be essential for transcriptional activation of calreticulin following UPR induction.

**ATF6n activates the calreticulin promoter through both ERSE1 and ERSE2**

The nuclear form of ATF6 orchestrates the induction of target genes during the UPR [35]. The presence of a putative ATF6-binding site in ERSE1 suggested ATF6n to be involved in calreticulin
activation. We transfected various constructs of the calreticulin promoter together with a plasmid encoding human ATF6n into U937 cells (Fig. 3E, F, G). We found that ATF6n activated the –298 nt construct up to 6.2-fold. ATF6n-mediated activation again showed a two-step pattern (Fig. 3E). Specific mutation of the CAAT motifs in both ERSE1 and ERSE2 (M4) completely abolished ATF6n-mediated activation (Fig. 3F). Mutation of the putative binding site for ATF6 in ERSE1 (M2) reduced the activating potential to that observed for ERSE2 alone. Mutation of the putative YY1-binding site in ERSE1 (M3), again, had no effect on ATF6n-mediated activation. In contrast, mutation of the YY1 site in ERSE2 (M6) abolished ATF6n-mediated activation of ERSE 2 (Fig. 3G). XBP1s and TFII-I, transcription factors reported to activate promoters through ERSE motifs, failed to affect ATF6n-mediated activation (data not shown). These results indicate that the two ERSE sequences of the calreticulin promoter are targeted by ATF6, by at least one CAAT binding protein, and (at least for ERSE2) by YY1.

**YY1 is required for ATF6 binding to ERSE 2, but not to ERSE 1 of the calreticulin promoter**

We performed EMSA in H1299 cells to identify the factors binding to the two ERSE motifs. We observed two binding activities (complex 1 and 2) in each motif (Fig. 3E). Specific enrichment analyses with specific antibodies identified complex 1 as YY1 (lanes 7 and 16 in Fig. 4A), and complex 2 as NFYA, the DNA-binding subunit of the tripartite transcription factor NFY (lanes 3 and 12). Specific mutations of
the CAAT-motif (M1 in ERSE 1 and M5 in ERSE 2) abolished NFYA binding, and no binding activity of complex 2 was observed with mutations of the YY1-binding site (M3 in ERSE 1 and M6 in ERSE 2). These results suggest that both NFYA and YY1 are directly binding to both ERSE sequences.

The demonstration of ATF6 binding by EMSA is technically challenging [30, 35]. Using nuclear extracts from H1299 cells transfected with the expression plasmid encoding ATF6n, a third complex was detectable binding to both ERSE motifs of the calreticulin promoter (Figs. 4B and C). The addition of a specific antibody against ATF6 shifted complex 3 in ERSE 1 (lane 4 in Fig. 4B), and abolished binding of complex 3 to ERSE 2 (lane 4 in Fig. 4C). ATF6n binding to ERSE 1 was undetectable when the CAAT-motif (M1) or the putative ATF6 binding site (M2) were mutated. Interestingly, mutating the YY1 binding site (M3) had no effect on ATF6n binding. Binding of ATF6n to ERSE 2 was abolished, when the CAAT-motif (M5) or the YY1 binding site (M6) were mutated. Reducing the nine nucleotides spacer sequence of the tripartite ERSE motif to 7 nucleotides (M8) also prevented ATF6n binding. Mutation of two nucleotides within the spacer region disrupting YY1 binding (M7) also decreased ATF6n binding. We thus conclude that NFYA (and also YY1 for ERSE2) are essential for ATF6n binding to the two ERSE sequences in the calreticulin promoter.
NFYA, YY1 and ATF6 binding to the calreticulin promoter is activated after induction of ER-stress

In order to assess changes in transcription factor binding to the ERSE sequences following induction of ER-stress, we performed chromatin immunoprecipitation experiments (Fig. 5). Again, K562 cells were treated with 300 nM thapsigargin. We observed that binding of NFY and YY1 to the ERSE sequences of the calreticulin promoter was detectable in untreated cells, whereas ATF6 binding was not present. After activation of ER-stress, NFY and YY1 binding was induced, and ATF6 became detectable. We conclude that binding to the calreticulin promoter of all three factors is induced following ER-stress.

Conditional expression of calreticulin in myeloid cells suppresses CEBPA translation

We have reported that calreticulin in vitro leads to a translational block of the myeloid key transcription factor CEBPA through enhanced binding to a stem loop structure within the CEBPA mRNA [15]. To assess the effect of calreticulin activation on CEBPA protein levels following ER-stress, we established a cell line model conditionally expressing HA-tagged calreticulin from a
tetracycline-responsive promoter in the myeloid U937 cell line. Twenty-four single cell clones were tested, and clone #8 was selected. All experiments mentioned subsequently were repeated with at least one different clone. We found a 10.8-fold increase of calreticulin mRNA after 3 days of tetracycline withdrawal (Fig. 6A). In contrast, CEBPA mRNA remained unchanged (Fig. 6B). Induction of calreticulin expression strongly increased binding of calreticulin protein to a ribooligonucleotide probe encoding the stem loop sequences of the CEBPA mRNA in a UV-cross link assay (Fig. 6C). Increased binding of calreticulin protein to CEBPA mRNA induced a decrease of CEBPA protein to below 30% (Fig. 6D). Finally, AML patients with activated UPR (S+/H11001) and thus induced calreticulin expression had hardly detectable CEBPA protein expression as compared to S- patients which expressed higher levels of CEBPA and lower calreticulin protein (Fig. 6E). These data indicate that increased expression of calreticulin in leukaemic cells blocks translation of CEBPA mRNA.

**Discussion**

In this report, we identified activation of the unfolded protein response (UPR) in a subgroup of AML patients. Our observation is based on the detection of the spliced variant of XBP1 mRNA in 16 out of 92 AML patients. XBP1s is produced after activation of the IRE1 protein through sensing of unfolded proteins in the lumen of the ER [23, 36, 37], and the detection of XBP1s was identified as a sensitive marker for activated UPR in AML patients. We found that patients with activated UPR also expressed higher levels of calreticulin and GRP78. Induction of these ER chaperones is pivotal for cell survival by re-establishing correct folding of proteins and preventing their aggregation. GRP78 expression is up-regulated in a variety of human tumours including breast, lung, liver and prostate cancer, whereas its expression in haematological malignancies has not been investigated so far.
The conditions activating the UPR in leukaemic cells remain to be elucidated. Cancer cells in general are susceptible to ER-stress because of intrinsic or extrinsic factors. In solid tumours, an environment is often created characterised by glucose deprivation, acidosis and hypoxia leading to the accumulation of underglycosylated and misfolded proteins in the ER, which triggers the UPR [38]. In contrast, intrinsic factors initiating the UPR might be more relevant in AML cells. Candidate events are mutations in secretory proteins, which lead to their misfolding as reported in patients with severe congenital neutropenia [39].

We confirmed calreticulin as a key target of the UPR. Beside its role in proper folding of glycoproteins in the ER [40], calreticulin was previously identified as an RNA-binding protein thereby blocking the translation of specific target mRNAs such as CEBPA, CEBPB and CDKN1A [34, 41]. In fact, we have previously found that calreticulin is activated in certain subtypes of AML with consecutive suppression of CEBPA protein [15, 16]. Consistent with this model, we were able to demonstrate the translational block of CEBPA protein through increased calreticulin expression in a conditional cell line model. Given the importance of suppressed CEBPA function for leukemogenesis [2, 4–16], we thus conclude that activation of the UPR with induction of calreticulin and thus CEBPA suppression may represent a significant event in AML with activated UPR.

The increased expression of calreticulin under ER-stress is mediated through the ATF6 pathway of the UPR. Two cis-acting ERSE in the proximal calreticulin promoter are crucial for promoter activation following ER-stress. NFYA binding to both ERSE motifs was essential for ATF6n-mediated activation of the calreticulin promoter. Interestingly, ATP6n bound directly to ERSE1 independent of YY1, whereas ERSE 2 has no direct binding site for ATF6n. An intact YY1 binding site was identified to be necessary for ATF6n binding to ERSE 2. YY1, NFYA and ATF6 binding to the calreticulin promoter is increased after induction of ER-stress in ChIP experiments. This is consistent with a report on activation of the GRP78 promoter under ER-stress conditions, where ATP6n is recruited to the ER-stress response element via interaction with YY1 [30].

Misfolded proteins in the ER are usually translocated to the cytoplasm, ubiquitinated and targeted to proteasomal degradation. However, accumulation of proteins in the cytoplasm blocks the efflux of unfolded proteins from the ER and leads to an increase of misfolded proteins in the ER lumen inevitably inducing the UPR and ultimately apoptosis of the cell. Our findings of activated UPR in malignant cells of AML patients might provide a basis for treatment strategies in AML. Multiple myeloma cell lines with activated UPR are especially prone to undergo apoptosis after treatment with the proteasome inhibitor bortezomib [42]. Also, high expression of calreticulin in patients with light chain amyloidosis predicts responsiveness to treatment with the alkylating agent melphalan [43]. Moreover, toxicity of melphalan appears to be increased in vitro in cells pretreated with ER-stress inducing agents [44]. These reports suggest increased responsiveness to chemotherapy in malignant cells with activated UPR. In particular, this study might provide a rationale for the use of bortezomib alone or together with standard regimens in AML patients presenting with an activated UPR in the leukaemic cells at diagnosis.

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Disclosures

All authors declare that they have no competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The sensitivity of the PCR assay was determined using pCDNA3 plasmids encoding wild-type human XBP1 (U) or the spliced form of XBP1 (S). A competitive PCR is depicted using constant amounts of wild-type XBP1 plasmid combined with increasing amounts of the spliced form of XBP1. The ratio observed with 30 ng of wild-type XBP1 plasmid and 20 ng of the spliced form of XBP1 was determined to separate AML patients with induced (S) versus uninduced (U) XBP1s mRNA.

Fig. S2 No difference was observed by RT-PCR of CEBPA mRNA expression between UPR positive AML patients and UPR negative AML patients (median 1.4 versus 1.9; P = 0.27). The median of AML-M0 patients served as a reference (1-fold). Boxes represent interquartile ranges, horizontal bars represent medians and thin bars represent the 10th and 90th percentiles.

Table S1 Sequences of the oligonucleotides used for EMSA.

Table S2 Clinical characteristics of patients

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