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MicroRNA-3662 expression correlates with antiviral drug resistance in adult T-cell leukemia/lymphoma cells

Kiyoshi Yasui [a, b], Mai Izumida [a, c], Takeya Nakagawa [d], Yoshinao Kubo [a, e], Hideki Hayashi [a], Takashi Ito [d], Hiroaki Ikeda [b], Toshifumi Matsuyama [a, f, *]

[a] Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan
[b] Department of Oncology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan
[c] Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, 852-8523, Japan
[d] Department of Biochemistry, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan
[e] Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
[f] Department of Cancer Stem Cell Biology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan

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ABSTRACT

Interferon regulatory factor (IRF) 4 and the proto-oncogene c-Rel cooperate in growth and antiviral drug resistance of adult T-cell leukemia/lymphoma (ATLL). To elucidate the target of IRF4 and c-Rel in ATLL, we determined the simultaneous binding sites of IRF4 and c-Rel using ChIP-seq technology. Nine genes were identified within 2 kb of binding sites, including *MIR3662*. Expression of miR-3662 was regulated by IRF4, and to a lesser extent by c-Rel. Cell proliferation was inhibited by knockdown of miR-3662 and expression of miR-3662 was correlated with antiviral drug resistance in ATLL cell lines. Thus, miR-3662 represents a target for therapies against ATLL.

1. Introduction

ATLL is a generally fatal malignancy. Prognosis is poor, and treatment strategies have traditionally focused on antiviral therapy with zidovudine (AZT) and interferon-alpha (IFN-α), as well as combination chemotherapy. However, most ATLL patients fare poorly under either regimen. In particular, management of relapsed, or primary refractory ATL is a challenge and needs more information about the mechanisms that govern the pathogenesis of this fatal disease [1].

In ATLL patients and cell lines, expression of IRF4 is elevated [2,3]. The IRF proteins, IRF1–IRF9, were originally identified as transcriptional regulators of type I IFN. IRF4 is a transcription factor that plays critical roles in lymphoid development and regulation of the immune response [4–7]; however, IRF4 binds DNA weakly due to its carboxy-terminal auto-inhibitory domain. It is thought that cooperative binding with IRF4-interacting proteins increases binding affinity [6], allowing IRF4 to regulate downstream genes. For example, FK506-binding protein 52 (FKBP52) binds to IRF4 in HTLV-1 (human T-cell leukemia virus type 1)–infected T cells, and represses IRF4 function by preventing its nuclear translocation and binding to target DNA [8]. IRF4-dependent IL-4 induction in the presence of PMA and ionomycin is enhanced by NFATc2 (NFAT1), and is functionally important in T-helper cell (Th) differentiation [9]. NFATc1 (NFAT2) also activates the human IL-2 and IL-4 promoters in cooperation with IRF4 [10]. Previously, we used a TAP tag to isolate IRF4-binding proteins from HTLV-1–infected T-cells; this analysis identified c-Rel, as a novel IRF4-associated protein [11].

c-Rel is a member of the nuclear factor κB (NF-κB) transcription factor family. Unlike other NF-κB proteins, which are expressed in a variety of cell types, c-Rel is expressed at high levels primarily in B and T cells, and many c-Rel target genes are involved in lymphoid cell growth and survival. Furthermore, expression of c-Rel was up-regulated in IFN/AZT resistant ATLL patient cells [12].

We hypothesized that IRF4 and c-Rel coordinately regulate the transcription of ATLL-related gene(s), and tried to determine their binding site using ChIP-seq technology.

* Corresponding author. Department of Cancer Stem Cell Biology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan.
E-mail address: tosim@nagasaki-u.ac.jp (T. Matsuyama).

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2. Materials and methods

2.1. Cells and chemicals

Jurkat cells, the ATLL-derived cell line ST-1, and MT-2, derived from human cord leukocytes co-cultured with ATLL cells, were cultured in RPMI 1640 (Wako, JP) supplemented with 10% fetal bovine serum (SIGMA, St louis, MO, USA) at 37 °C in a 5% CO_2 atmosphere. Peripheral blood mononuclear cells (PBMCs) were separated from blood of healthy voluntary donors by Ficoll-Paque™ PLUS (GE Healthcare) density gradient centrifugation. T cells were isolated from PBMCs using CD3 MicroBeads, human (Miltenyi Biotec). IFN-α (Sumitomo Dainippon Pharma) and AZT (Cayman Chemical) were used for antiviral drug resistance assays.

2.2. ChIP-seq

ST-1 cells were cross-linked with formaldehyde (final concentration, 1%) for 10 min. The reaction was quenched with glycine, and cell lysates were sonicated using an ULTRASONIC CELL DISRUPTOR (Microson, Barcelona, Spain). DNA fragments were immunoprecipitated with anti-IRF4(Santa Cruz Biotechnology, sc-6059) or anti–c-Rel (Santa Cruz Biotechnology, sc-70) antibody. Sample preparation for sequencing was performed according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequences were aligned to the human hg19 reference sequence, and peak calling was performed using the DDBJ pipeline.

2.3. Real-time PCR

Total RNA was prepared using the miRNeasy Mini Kit (Qiagen, Venlo, Netherlands). For IRF4, c-Rel, and β-actin, reverse transcription was conducted for 30 min at 42 °C from 500 ng of purified total RNA using PrimeScript RTase (Takara), followed by 50 cycles of PCR (10 s denaturation at 95 °C, 1 min extension at 60 °C). The THUNDERBIRD® SYBR® qPCR Mix (TOYOBO) was used to monitor the PCR products on an ABI PRISM 7900HT. The PCR primers used for IRF4 were 5′-ATGCTTTGGAGAGGAGTTTC-3′ and 5′-CTGGATTGCTGATGTGTTC-3′, those for c-Rel were 5′-CAGAGGGGAATGCGTTTTAG-3′ and 5′-CCGTCTCTGCAGTCTTTTCC, those for β-actin were 5′-AAGAGAGGCATCCTTCACCCT-3′ and 5′-TACATGGCTGGGGTGTTGAA-3'.

2.4. Immunoblot analysis

Cell pellets were lysed in sample buffer. Proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and incubated with the indicated antibodies. Specific proteins were visualized with the appropriate HRP-conjugated antibodies and the ECL Plus detection system.

3. Results and discussion

3.1. Candidate gene(s) regulated by IRF4 and c-Rel

To detect candidate gene(s) potentially regulated by IRF4 and c-Rel, we carried out ChIP-seq analysis using ST-1 cells. A total of 236 IRF4 and c-Rel binding sites overlapped (Fig. 1A). Thirty-two of these sites contained well-known IRF binding motifs (Fig. 1B), and 9 of the 32 mapped within 2 kb region of predicted genes (Fig. 1C). One of the nine genes was *MIR3662* (Fig. 1D), which encodes miR-3662, originally identified as an miRNA that is upregulated in patients with lung cancer [13]. Another group showed that miR-3662 inhibits leukemogenesis [14]. Given the association of miR-3662 with tumorigenesis and leukemogenesis, we postulated that overexpression of miR-3662 under the control of IRF4 and c-Rel contributes to ATLL onset and antiviral drug resistance.

3.2. Effect of miR-3662 in ATLL cell lines

To test this hypothesis, we compared expression of mir-3662 between ATLL cell lines. As shown in Fig. 2A, expression of miR-3662 was 5-fold higher in an ATLL cell line than in the T-cell control ($CD3^+$PBMC, Jurkat). This observation indicates that miR-3662 expression is involved in the pathological mechanism of ATLL. Furthermore, expression of miR-3662 was well correlated with expression of IRF4 and c-Rel (Fig. 2B–D).

3.3. Effect of IRF4 on expression of miR-3662

To determine whether miR-3662 expression is controlled by IRF4 and c-Rel, we first confirmed that IRF4 and c-Rel bound to the proximal region of *MIR-3662 in vivo*, using ATLL-derived cell line ST-1. For this purpose, we performed ChIP assays in ST-1 cells using specific PCR probes. Anti-IRF4 antibody specifically precipitated the proximal region of *MIR-3662* (Fig. 3A). Anti–c-Rel antibody also specifically precipitated a fragment of the gene, but the interaction was weaker than that of IRF4 (Fig. 3A), possibly because c-Rel is not as highly expressed in ST-1 cells (Fig. 2B, D). As described above, IRF4 is thought to play a more important role in the expression of miR-3662 than c-Rel in ST-1 cells. Therefore, to confirm that expression of miR-3662 is governed by IRF4, we used a Tet-off system in which IRF4 expression was controlled by doxycycline (Dox) (Hayashi unpublished Data). Dox treatment decreased expression of IRF4 at both the mRNA and protein levels (Fig. 3B and C), and concomitantly decreased the expression of miR-3662 (Fig. 3D). Taken together, these results suggest that expression of miR-3662 is controlled by IRF4, whereas the involvement of c-Rel remains to be determined.

3.4. Effect of miR-3662 on cell growth

It is conceivable that IRF4 promotes the proliferation of ATLL cells by driving the expression of miR-3662. To test this hypothesis, we knocked down miR-3662 in ST-1 cells. Indeed, the knockdown cells grew slowly than the wild type (Fig. 4A).

IKKβ, a component of the NF-kB signaling pathway, is a target of miR-3662 [14]. Signaling via NF-kB occurs via two pathways, canonical and non-canonical. The canonical pathway mediates inflammatory responses, whereas the non-canonical pathway is involved in immune cell differentiation and maturation and secondary lymphoid organogenesis. In the canonical pathway, IKKβ is an essential component of the NF-κB pathway that phosphorylates IκBα, causing NF-κB to translocate into the nucleus. In the ATLL cell line, overexpression of miR-3662 is expected to reduce the expression of IKKβ and thus inhibit the canonical pathway. Indeed, miR-3662 knockdown in ST-1 cell induced phosphorylation of IκBα (data not shown). However, although the canonical NF-κB pathway was inactivated, proliferation was activated. A similar phenomenon has been observed for HBZ, the only viral gene constitutively expressed in ATLL cells: HBZ inhibits the canonical pathway of NF-κB, but promotes T-cell proliferation [15]. In addition, miR-31 expression is also decreased in ATLL patients [16]; this miRNA targets NIK, which plays an important role in activation of the non-canonical pathway. Despite the fact that further studies are required for other factors targeted for miR-3662, these results indicate that the relative activation of the non-canonical pathway is elevated in ATLL cells.