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Abstract: Adult T-cell leukemia (ATL) is an aggressive disease characterized by visceral invasion, and ATL regulates matrix metalloproteinase (MMP) activities of the endothelial cells. The controlling system of MMP activities in ATL is regulated by various factors such as Emmprin and tissue inhibitor of MMP. In this study, we demonstrated that Testican 3 expression in ATL decreased the activity of MMP. Furthermore, we showed that the expression of Testican 3 was regulated by activating transcriptional factor 3 in human T-lymphotropic virus type I-related cell lines. Thus, Testican 3 is a novel regulator to reduce the activity of MMP in ATL.

Testican 3 expression in adult T-cell leukemia

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Abstract

Adult T-cell leukemia (ATL) is an aggressive disease characterized by visceral invasion, and ATL regulates matrix metalloproteinase (MMP) activities of the endothelial cells. The controlling system of MMP activities in ATL is regulated by various factors such as Emmprin and tissue inhibitor of MMP. In this study, we demonstrated that Testican 3 expression in ATL decreased the activity of MMP. Furthermore, we showed that the expression of Testican 3 was regulated by activating transcriptional factor 3 in human T-lymphotropic virus type I-related cell lines. Thus, Testican 3 is a novel regulator to reduce the activity of MMP in ATL.

Keywords adult T-cell leukemia, human T-lymphotropic virus type I, activating transcriptional factor 3, Testican 3

Introduction

Vascular infiltration of tumor cells through the endothelial cells is a critical step of cancer invasion and metastasis, which is an important prognostic factor of malignant diseases. In this process of neoplasm infiltration, production of matrix metalloproteinases (MMPs) from vascular endothelial cells is increased [1]. In human T-lymphotropic virus type I (HTLV-I)-related diseases, malignant cells express MMP-13 activating protein, Emmprin, which activates MMP-2 [2]. Co-culture of fibrocytes with HTLV-I related cell line MT-2 showed high expression of MMP-2 when MT-2/fibrocyte ratio was 0.1, whereas the activity of MMP-2 was decreased when MT-2/fibrocyte ratio was 1.0 [2]. As MT-2 increases MMPs activity of fibrocytes, high MT-2/fibrocyte ratio should result in high MMPs secretion. Therefore, we hypothesized that MT-2 secretes not only MMP activating Emmprin but also MMP inhibiting factors. To evaluate the difference in MMP activity between co-cultured human aortic endothelial cells (HAECs) with MT-2 and adult T-cell leukemia (ATL) derived cell line MT-1, cDNA subtraction analysis was performed using MT-2 and MT-1.

Materials and Methods

Cells Culture

HTLV-I-transformed cell lines MT-2 and MT-4, ATL-derived cell lines MT-1 and ATL-1K, and HAECs (KURABO, Japan) were cultured in conditions of RPMI medium 1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) at 37°C in as humidified atmosphere containing 5% CO_2.

Gelatin zymography

HAECs (1×10^6) were seeded on 6-well culture dish and cultured for 2 days. Culture media were removed before co-culture with the HTLV-I related cell lines. HTLV-I related cell lines (1×10^5 or 1×10^6) were added and cultured for 2 days with FBS-free RPMI1640 culture media. Supernatants were collected and used for zymography analysis [2]. The gelatinolytic activity of MMP-2 and MMP-9 in the co-cultured supernatants were evaluated using gelatin zymography by the methods of supplier (Cosmo bio, Japan). In brief, after 30 hour-incubation at 37 °C in developing buffer, the gel was stained with 0.1 % Coomassie Brilliant Blue R250. For analyses of gelatinolytic activity of MMP-2 and MMP-9, gels were scanned and were imported into Adobe Photoshop software (v. 7.0, Adobe System).

RNA isolation and cDNA subtraction

Total cellular RNA was isolated with the TRIzol reagent (Invitrogen, USA), and DNA contamination was removed by DNase treatment. cDNAs of MT-1 and MT-2 were formed by SMART PCR cDNA Synthesis Kit (Clontech, Japan) and then cDNA subtraction was performed by PCR-Select cDNA Subtraction Kit (Clontech, Japan) according to the methods of supplier [4]. After cDNA subtraction, PCR product was cloned in *E. coli* DH5-α with pCR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen, USA). Plasmid DNA from DH5-α was isolated and sequencing reaction was carried out by Big Dye Terminators v1.1 Cycle Sequencing Kit (Applied Biosystems, UK), and then analyzed by ABI 310 Genetic Analyzer (Applied Biosystems, UK). Searches with the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) were conducted for all sequences to analyze unknown sequences detected with the cDNA subtraction method.

Reverse-transcription (RT)-PCR

Peripheral blood mononuclear cells (PBMCs) of a healthy donor and leukemia cells of 8 patients with acute leukemia were prepared by Lymphoprep (Daiichi Pure Chemicals, Japan) and total RNAs were extracted. Two samples of non-Hodgkin lymphoma

(NHL) and 5 samples of ATL were collected by biopsy or necropsy from pathological lymph nodes, and total RNAs were extracted from these samples. Informed consent was obtained from all patients and healthy donor. cDNA was synthesized from 400 ng of total cellular RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Japan). The cDNA was then amplified by PCR using Blend Taq Plus DNA polymerase (TOYOBO, Japan). Amplification of the housekeeping gene ribosomal RNA L7a was used to verify RNA quality and RT-PCR techniques. Expression of L7a, Testican 3 (GenBank accession number AJ001454; Maurer, 1997) and ATF3 were examined by RT-PCR as described previously [5] using the following PCR primers; L7a forward primer, 5'- ATGGGTCAGAAGGATTCCTA -3'; L7a reverse primer, 5'- CGTCACACTTCATGATGGA -3'; Testican 3 forward primer, 5'- CTCAAGATTCTCAGACTGCA -3'; Testican 3 reverse primer, 5'- GAGCTCTGACTGGTCCA -3'; ATF3 forward primer, 5'- CTCCTGGGTCACTGGTGTTT -3', ATF3 reverse primer, 5'- GTCGCCTCTTTTTCCTTCA -3'. Products size were 204 bp for L7a, 494 bp for Testican 3 and 204 bp for ATF3. Cycling condition was followed by 30 cycles consisting 94°C for 30 s, 57°C for 30 s and 72°C for 1.5 min. PCR products were electrophoresed in 2% agarose gels and visualized with ethidium bromide staining.

Western blotting of ATF3 and Testican 3

Protein extracts from MT-2 and 5 ATL patients were electrophoresed on SDS-12% polyacrilamide gel (SDS-12% PAGE) and blotted onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Pharmacia Biotech, USA). The blot was subsequently incubated with a rabbit anti-human-ATF3 antibody (Santa Cruz Biotechnology Inc, USA: dilution 1:200) and anti-rabbit IgG antibody-HRP (Santa Cruz Biotechnology Inc, USA: dilution 1:200). The blots were performed by chemiluminescence with ECL-Plus Western Blotting Detection System (Amersham Pharmacia Biotech, USA). For a standard control, anti-human-GAPDH antibody (Santa Cruz Biotechnology Inc, USA: dilution 1:200) was used. To detect Testican 3, goat anti-human-Testican 3 antibody (Santa Cruz Biotechnology Inc, USA: dilution 1:200) was used for the first antibody reaction, and donkey anti-goat IgG antibody-HRP was used for the second antibody (Santa Cruz Biotechnology Inc, USA: dilution 1:2000).

Target gene silencing by short interfering RNA (siRNA)

Stealth siRNA (Invitrogen, USA) targeting ATF3-HSS100777 was used for silencing

the gene [6]. siRNA sequence for silencing ATF3 is double strand RNA, AAUCUUAUUUCUUUCUCGUCGCCUC, and its complementary sequence. This double-stranded siRNA was transfected into MT-2 cells using Lipofectamine 2000 (Invitrogen; USA). In brief; 100 pmol of these double-stranded siRNA and 50 μl of Lipofectamine 2000 in 500 μl of Opti-MEM I Reduced-Serum medium (Invitrogen; USA) were mixed and incubated at room temperature for 20 min. The mixture was added to 2 ml of pre-culture Opti-MEM I serum-reduced medium with 1 x 10^6 MT-2 cells, and incubated at 37°C for 48 hours. Total RNAs were analyzed by RT-PCR.

Induction of ATF3 in HTLV-I-related cell lines

Anisomycin is a strong inducer of ATF3 to activate p38 or c-Jun N-terminal kinase pathway [7]. To induce ATF3, anisomycin (Calbiochem, USA) was added to the culture media of MT-1, MT-2, ATL-1K and MT-4 (final concentration 50 ng/ml). After 3 hours incubation, total RNA was extracted and RT-PCR was used for the expression of ATF3 and Testican 3.

Results

Gelatin zymography analysis of co-cultured HAEC with HTLV-I related cell lines

MT-1 (1 x 10^5) and HAECs (1 x 10^6), MT-1 (1 x 10^6) and HAECs (1 x 10^6), MT-2 (1 x 10^5) and HAEC (1 x 10^6), and MT-2 (1 x 10^6) and HAEC (1 x 10^6) were co-cultured for 2 days, and supernatants were collected. Gelatin zymography analyses were performed for these supernatants. Figure 1 shows 72 kDa band of pro-MMP-2 and 62 kDa band of active form of MMP-2 by zymogram. When HAEC was co-cultured with MT-1, zymogram showed high activity of MMP-2 at MT-1/HAEC ratio=1.0. However, when HAEC was co-cultured with MT-2, MMP-2 activity became lower by changing MT-2/HAECs ratio 0.1 to 1.0. Considering that MT-2 secreted MMP-13 activating Emmprin by contacting the endothelial cells, these results suggest that MT-2 also secreted some factors that inhibited MMPs activation.

cDNA subtraction of MT-1 and MT-2

After 2 days of FBS-free RPMI1640 culture, total RNA was extracted from MT-1 and MT-2. cDNA subtraction of these HTLV-I-related cell lines revealed sequences with different expression levels and those were analyzed by BLAST search (http://www.ncbi.nlm.nih.gov/blast/). Dominant sequence of MT-1 was CD99 mRNA and dominant sequences of MT-2 were ATF3 mRNA, Testican 3 mRNA and CD9 mRNA. RT-PCR was performed for ATF3 and Testican 3. MT-2 showed high

expression of Testican 3 and ATF3, but MT-1 showed only weak ATF3 expression (Fig. 2).

Analysis of ATF3 and Testican 3 expression

To detect ATF3 and Testican 3 expressions in hematological malignancies, RT-PCR was performed in 17 cell lines and 15 clinical samples of hematological malignancies. Figure 3A shows ATF3 and Testican 3 mRNA expression in cell lines of hematological malignancies; chronic myelogenous leukemia-derived cell lines (CML-C-2 and K562), acute myelogenous leukemia-derived cell lines (PL21 and NM4), Hodgkin lymphoma-derived cell line (HD70), B-cell lymphoma-derived cell line (SP49), B-cell acute lymphocytic leukemia-derived cell lines (BALL, NALL and PALL), T-cell acute lymphocytic leukemia-derived cell lines (TALL1 and TALL2), T-cell lymphoblastic cell lines (CEM and Jurkat), ATL-derived cell line (MT-1), HTLV-I-transformed cell line (MT-2), ATL-derived cell line (ATL-1K), and HTLV-I-transformed cell line (MT-4). MT-2 showed expression of Testican 3 mRNA and HTLV-I related cell lines showed high expression of ATF3. Figure 3B shows ATF3 or Testican 3 mRNA expression in clinical samples of hematological malignancies. RT-PCR revealed ATF3 and Testican 3 amplification from 4 AML, 1 CML blast crisis, 1 chronic

lymphocytic leukemia, 2 ALL, lymph nodes of 2 NHL, and lymph nodes of 5 ATL. Healthy human PBMCs was used for normal control. Five ATL samples showed highly expressed ATF3 and Testican 3 by RT-PCR. Highly expressed ATF3 and Testican 3 were observed in 5 lymph nodes of ATL patients by western blotting (Fig. 3C). These results indicate that ATF3 expression was related to Testican 3 expression in ATL lymph nodes.

ATF3 silencing with siRNA treatment

To evaluate the relation between the expression of ATF3 and Testican 3, ATF3 silencing was performed by Stealth siRNA ATF3-HSS100777. RT-PCR of ATF3 and Testican 3 were performed 48 hours after adding siRNA. siRNA effectively inhibited the expression of ATF3 (Fig. 4.A). The expression of Testican 3 was also reduced in ATF3 silenced MT-2. Co-cultured supernatant of 1×10^6 HAECs with 1×10^5 ATF3 silenced MT-2 showed high MMP-2 activities by gelatin zymography analysis (Fig. 4B).

ATF3 induction for HTLV-I related cell lines

ATF3 is a stress induced transcriptional factor and is related with various mitogen

activated protein kinase (MAPK) pathways. Anisomycin is a strong stimulator of MAPK that induces ATF3. Anisomycin (final concentration; 100 pmol) was added to MT-1, MT-2, ATL-1K and MT-4 in FBS-free RPMI1640. After 3 hours, RNAs were extracted, and RT-PCR for ATF3 and Testican 3 were performed (Fig. 5). Not only MT-2, but also MT-1 and MT-4 showed expression of ATF3 and Testican 3 by adding anisomycin. These results indicate that ATF3 induction might be related to Testican 3 expression.

Discussion

HTLV-I is the causative agent of ATL [8] and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM) [9]. In the early 1980s, Miyoshi *et al.* established 2 HTLV-I-infected cell lines, one was MT-1 of ATL cell origin [10] and another was MT-2 of HTLV-I-transformed cell line [11]. ATL cells influence MMP activities of the endothelial cells by cell-to-cell contact, and infiltrate by destroying basement membrane and tight junction [12]. The infiltration system must possess regulation mechanism that contains not only MMPs activation to break the basement membrane, but also MMP activities inhibition to retain malignant cells in the tissue. Among the cell lines of hematological malignancies, RT-PCR revealed that

Testican 3 expression was detected only in MT-2. In the clinical samples of hematological malignancies, Testican 3 was expressed in ATL patients, but not in other hematological malignancies. This is the first report of Testican 3 expression in ATL.

Testican 3 is one of the BM-40 matrix proteins mainly expressed in the brain [3]. Testican 3 is an MMPs inhibitor because glioma cells showed metastasis in the absence of Testican 3 expression [3]. Testican 3 inhibits the activation of MMPs by interrupting membranous type MMP [3]. In contrast, tissue inhibitor of metalloproteinase (TIMP) inhibits MMPs by interrupting MMPs-substrates interaction [13]. Moreover, many invasive neoplasm express membranous type MMP activating Emmprin [2]. We detected high expression of Emmprin in all the cell lines and clinical samples of hematological malignancies by RT-PCR (data not shown). These MMP regulating factors expressed in ATL cells might be related to each other and act to retain or invade local lesion of ATL. Considering that HTLV-I-infected cells of TSP/HAM remains in the central nervous system (CNS) for long time, Testican 3 expression in CNS could also be related to the progression of TSP/HAM. Although the functions of Testican 3 in ATL are not clear, Testican 3 may reduce infiltration of ATL cells by controlling MMPs activities. For ATL cells, to remain in the lymph nodes or subcutaneous lesions, Testican 3 must be an important factor for

tumorigenesis.

cDNA subtraction analysis also detected the difference of ATF3 expression between MT-1 and MT-2. MT-2 showed high expression of ATF3, but MT-1 showed weak expression of ATF3. Interestingly, western blot analysis in ATL patients showed that the intensity pattern of ATF3 and that of Testican 3 was proportionally, which indicate that ATF3 regulated the expression of Testican 3. Stress induced transcriptional factor ATF3 acts as a reducer of transcription [14]. There are many reports indicating that ATF3 expressions are influenced by MAPK pathway [7]. This is supported by the following results: p38 MAPK mainly influences expression of ATF3and results in apoptotic changes of mouse embryonic fibroblasts [7]; ATF3 forms heterodimer with other transcriptional factors and inhibits transcriptional activities of c-Jun [6]. Among hematological malignancies, Hodgkin lymphoma cells expressed high ATF3 [15]. Our study showed that ATF3 expression and Testican 3 expression was related in ATL. However, Testican 3 was not expressed in the clinical samples and cell lines of hematological malignancies except ATL or HTLV-I related cell lines. The existence of Tax and HTLV-I b-zip factor (HBZ) in HTLV-I-related disease could be unique additional factors not observed in other hematological malignancies. These are supported by the following results: Tax enhances the transcriptional activity of ATF3

[16] and HBZ reduces transcriptional activity of other transcriptional factors [17]. In our study, MT-2 showed no expression of Testican 3 when ATF3 was silenced, but MT-1 and MT-4 showed high expression of Testican 3 when ATF3 was induced by anisomycin. These results indicate that ATF3 regulates Testican 3 expression and is related to the tumourigenesis of ATL. Considering that ATF3 was highly induced by ultra-violet [18], Testican 3 regulated by ATF3 could retain the local growth of ATL cells in the subcutaneous lesion by inhibiting activities of MMP.

In conclusion, we provided the first evidence that Testican 3 was highly expressed in ATL, and Testican 3 regulated by ATF3 reduced MMP-2 activity in HTLV-I related cell lines. Thus, Testican 3 expression regulated by ATF3 could be related to the tumorigenesis of HTLV-I-associated diseases.

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Figure Legends

Figure 1

Stimulation of MMP-2 gelatinolytic activity in co-cultured HAECs with HTLV-I related cell lines. Gelatin zymography was performed with culture media collected from 2 days of culture. The numbers under the zymogram indicate the cell number of HTLV-I related cell lines and HAECs used in co-cultures. Bands at 72 and 62 kDa correspond to pro- and activated forms of MMP-2 respectively.

Figure 2

RT-PCR for mRNA expression of ATF3 and Testican 3 in MT-1 and MT-2 cells. A housekeeping gene ribosomal RNA L7a was used to verify RNA quality and RT-PCR techniques.

Figure 3

(A) ATF3 and Testican 3 expression in cell lines of various hematological malignancies.

(B) ATF3 and Testican 3 expression in clinical samples of hematological malignancies.

(C) Western blotting for ATF3 and Testican 3 in ATL.

Figure 4

(A) ATF3 silencing in MT-2. Wild type MT-2, MT-2 with ATF3 siRNA and MT-2 with control siRNA were cultured for 48 hours after treatment by siRNA. Amplified bands are for ATF3 and Testican 3, respectively. Lane 1: wild type MT-2, Lane 2: MT-2 with ATF3 siRNA, Lane 3: MT-2 with control siRNA

(B) Gelatin zymography analysis of HAECs co-cultured with ATF3 silenced MT-2. Lane 1: wild type MT-2 (1×10^6), lane 2: HAECs (1×10^6), lane 3: HAECs (1×10^6) with MT-2(1×10^5), lane 4: HAECs (1×10^6) with ATF3 silenced MT-2 (1×10^5), lane 5: HAECs (1×10^6) with control siRNA added MT-2 (1×10^5). Bands at 72 and 62 kDa correspond to pro- and activated forms of MMP-2 respectively.

Figure 5

Induction of ATF3 in HTLV-I related cell line. RT-PCR of ATF3 and Testican 3 was performed after anisomycin treatment for MT-1, MT-2, ATL-1K and MT-4. Anisomycin added MT-2 showed higher expression of Testican 3. MT-1 and MT-4 also showed expression of Testican 3 by adding anisomycin.

Figure

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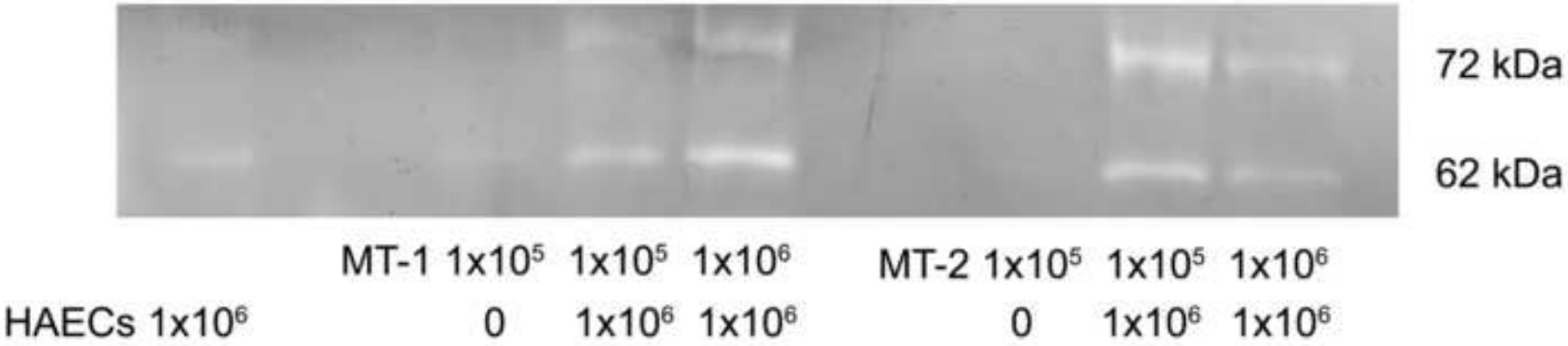

Figure 2

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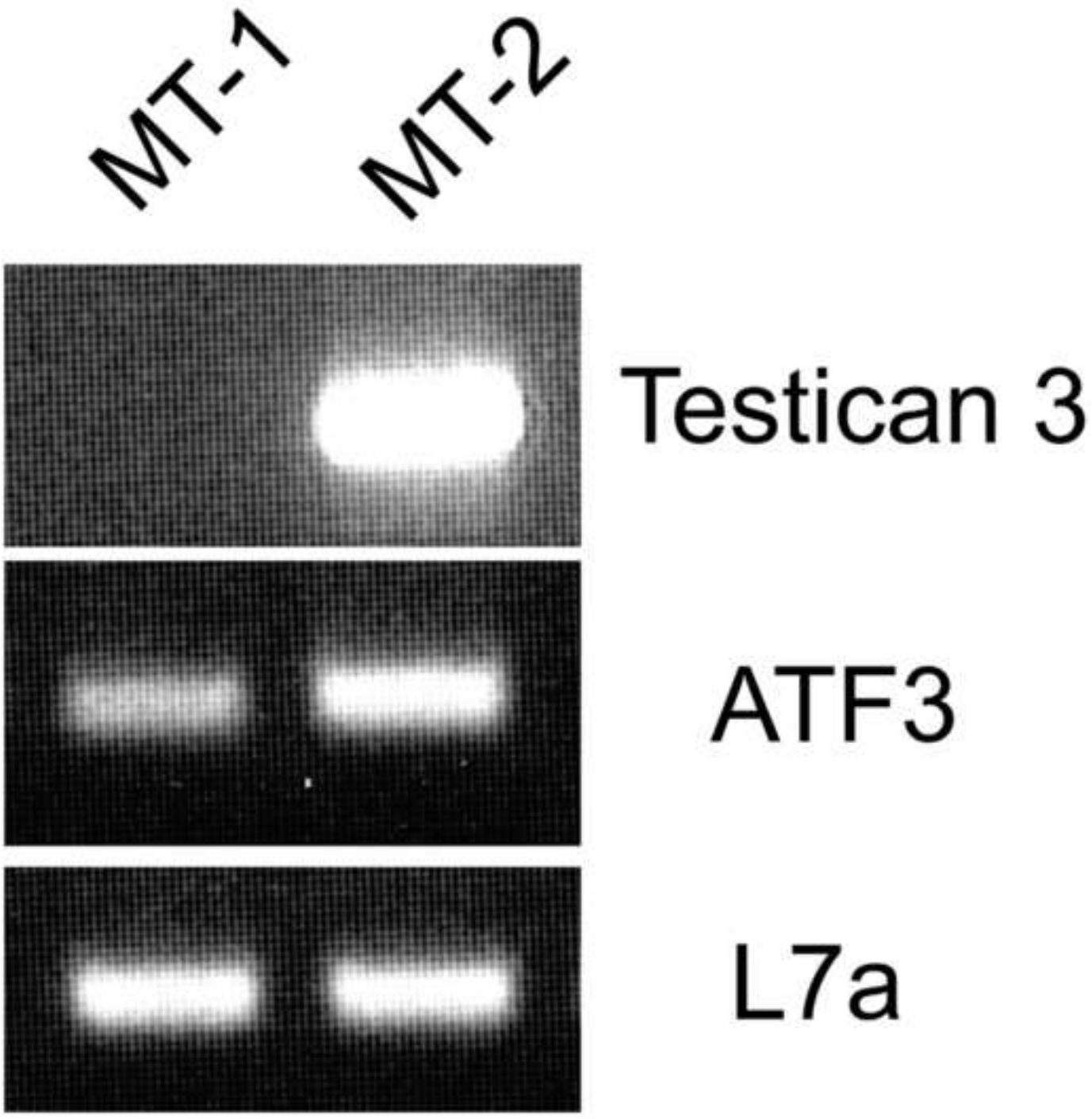

Figure 3A

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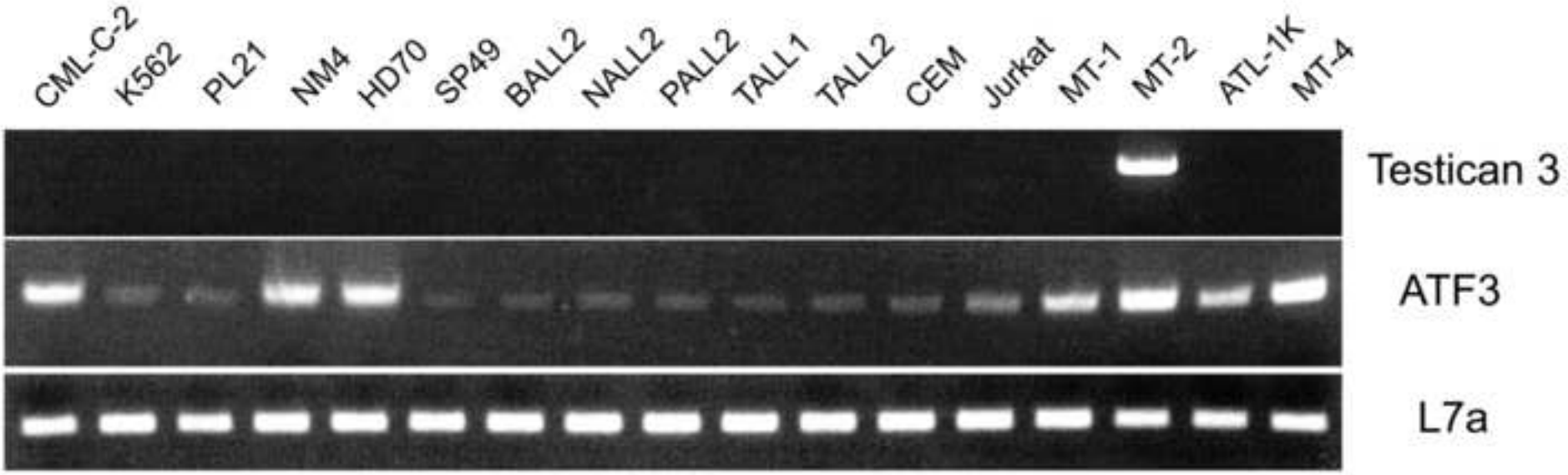

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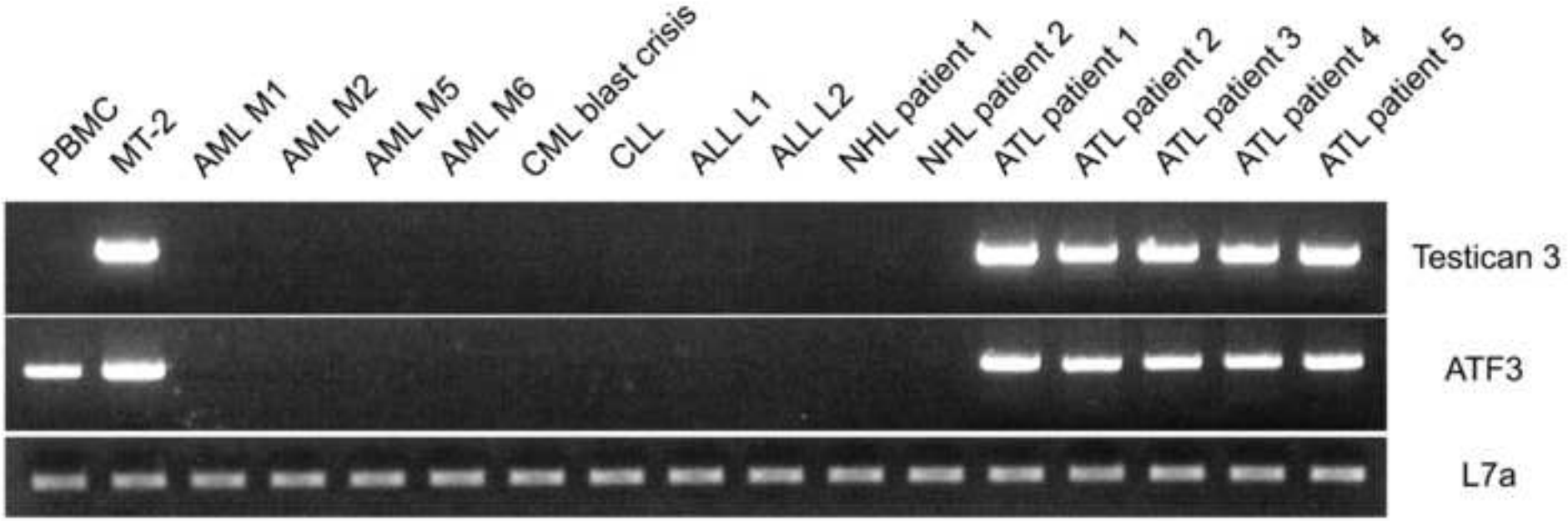

Figure 3C

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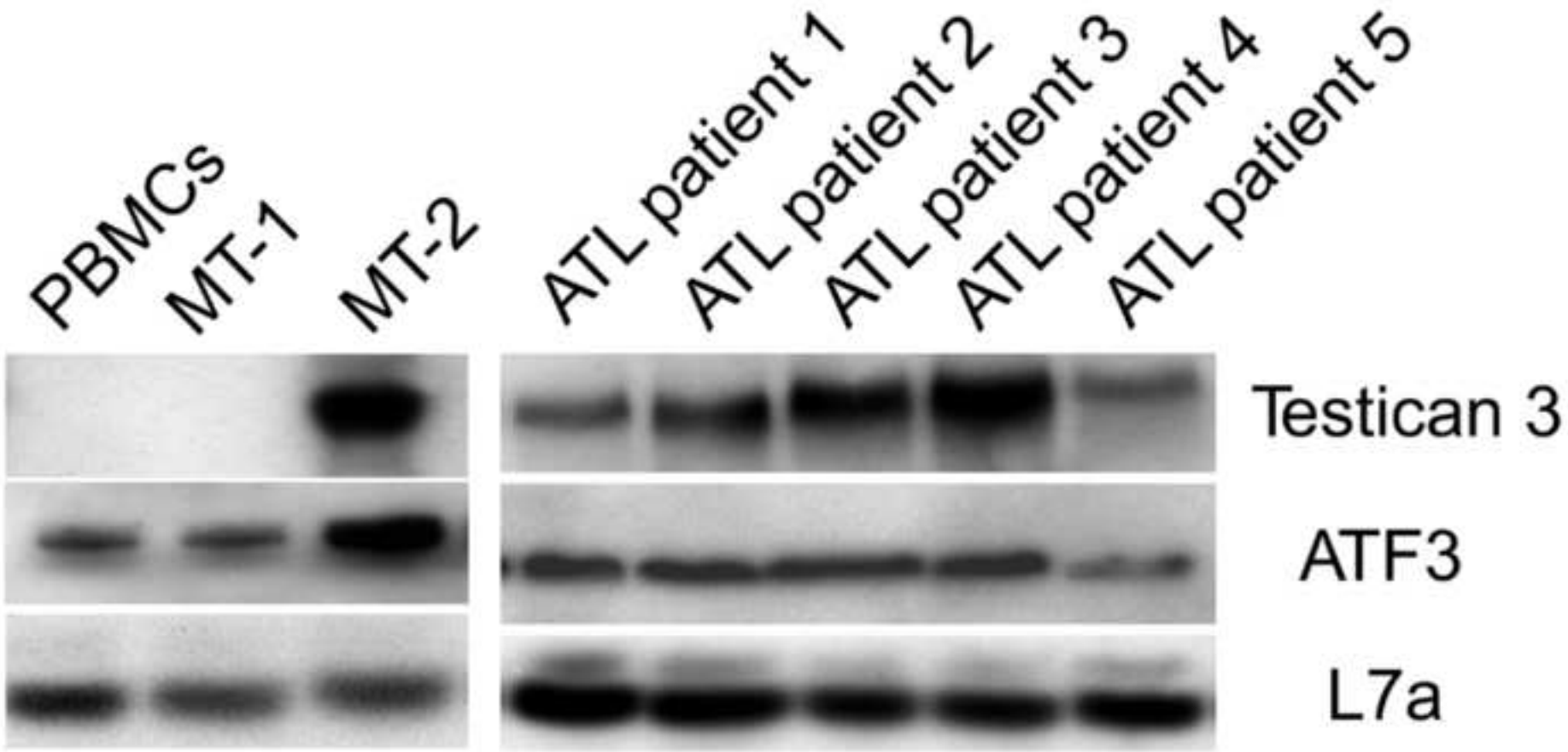

Figure 4A

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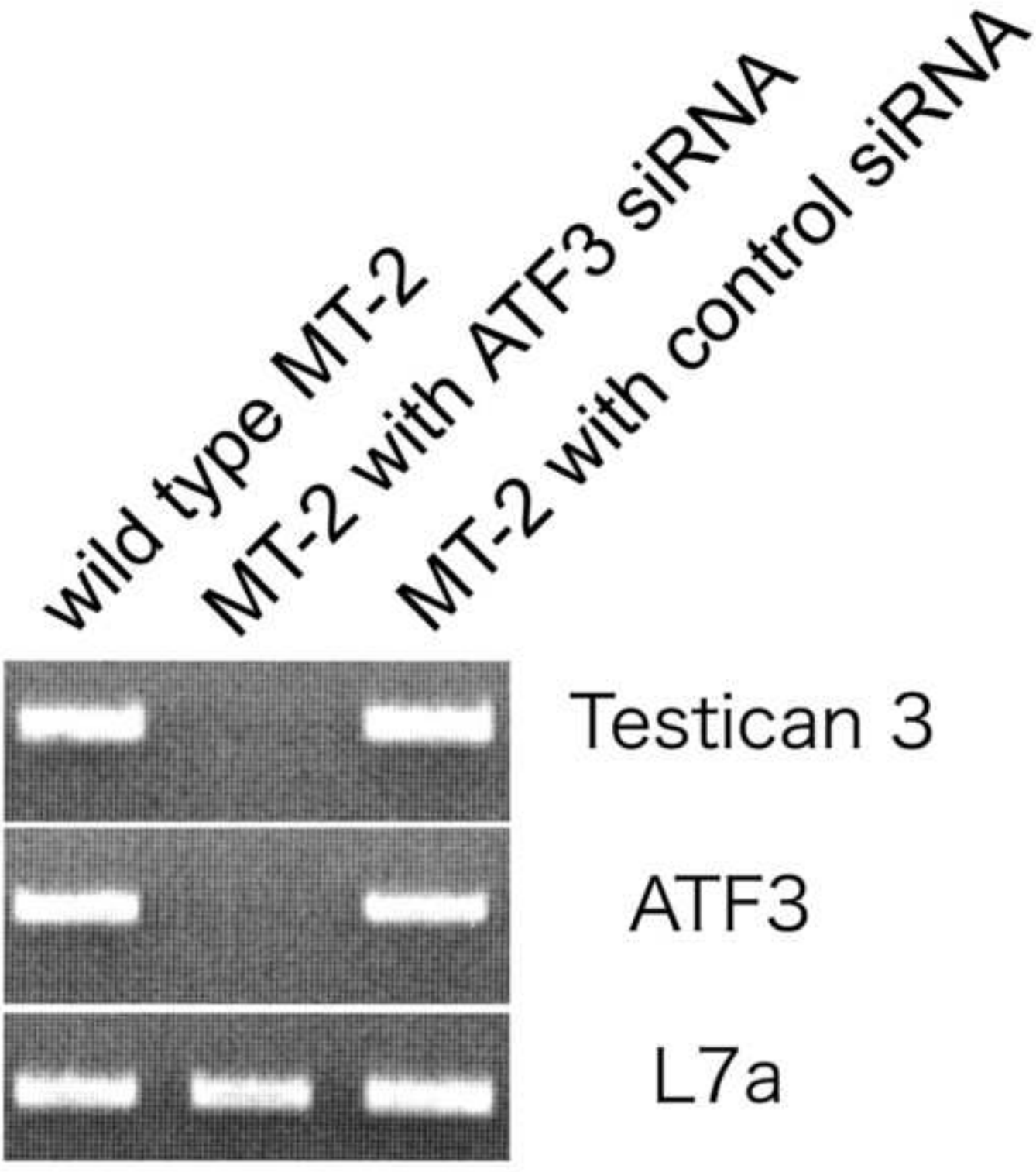

Figure 4B

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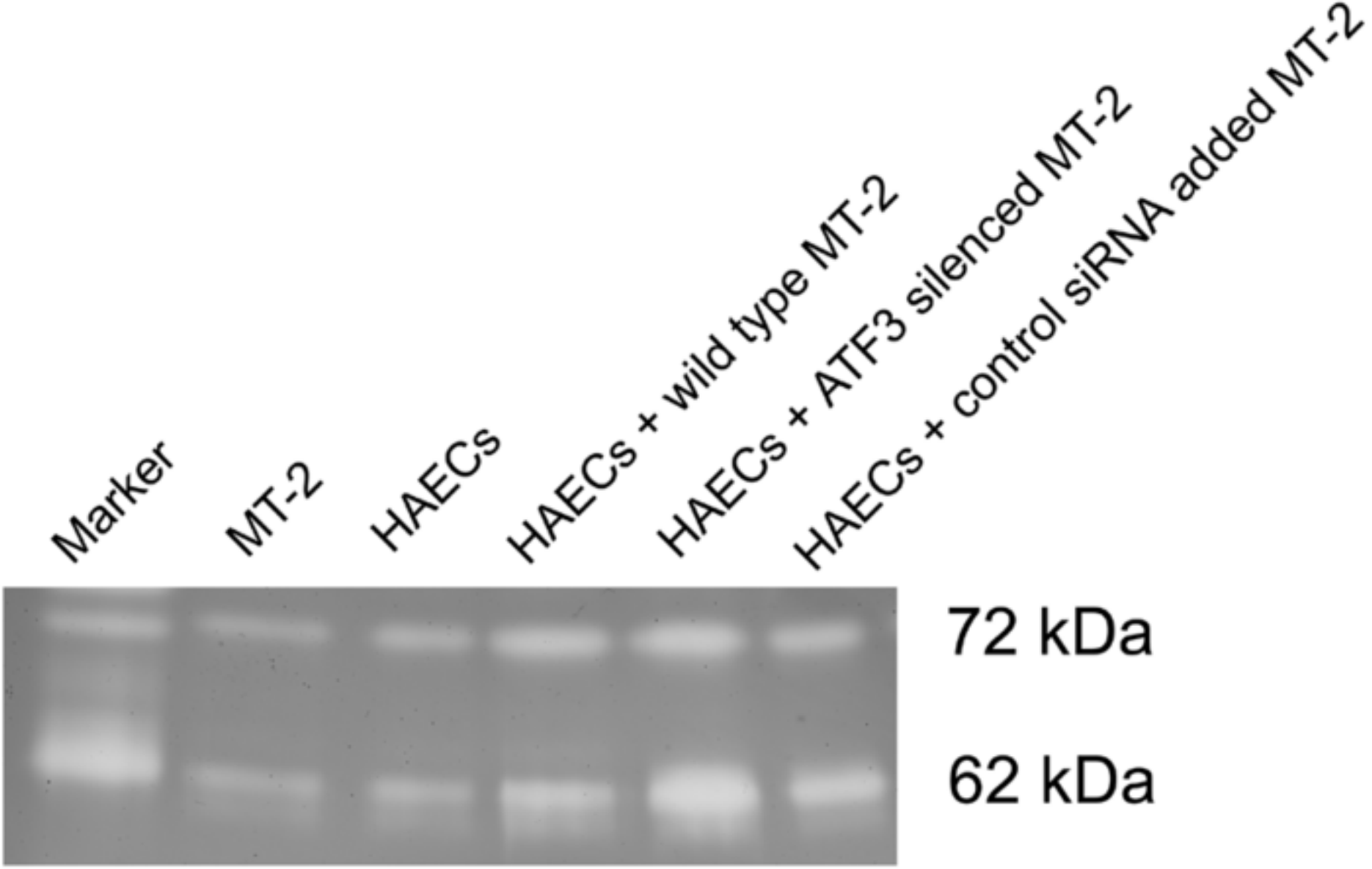

Figure 5

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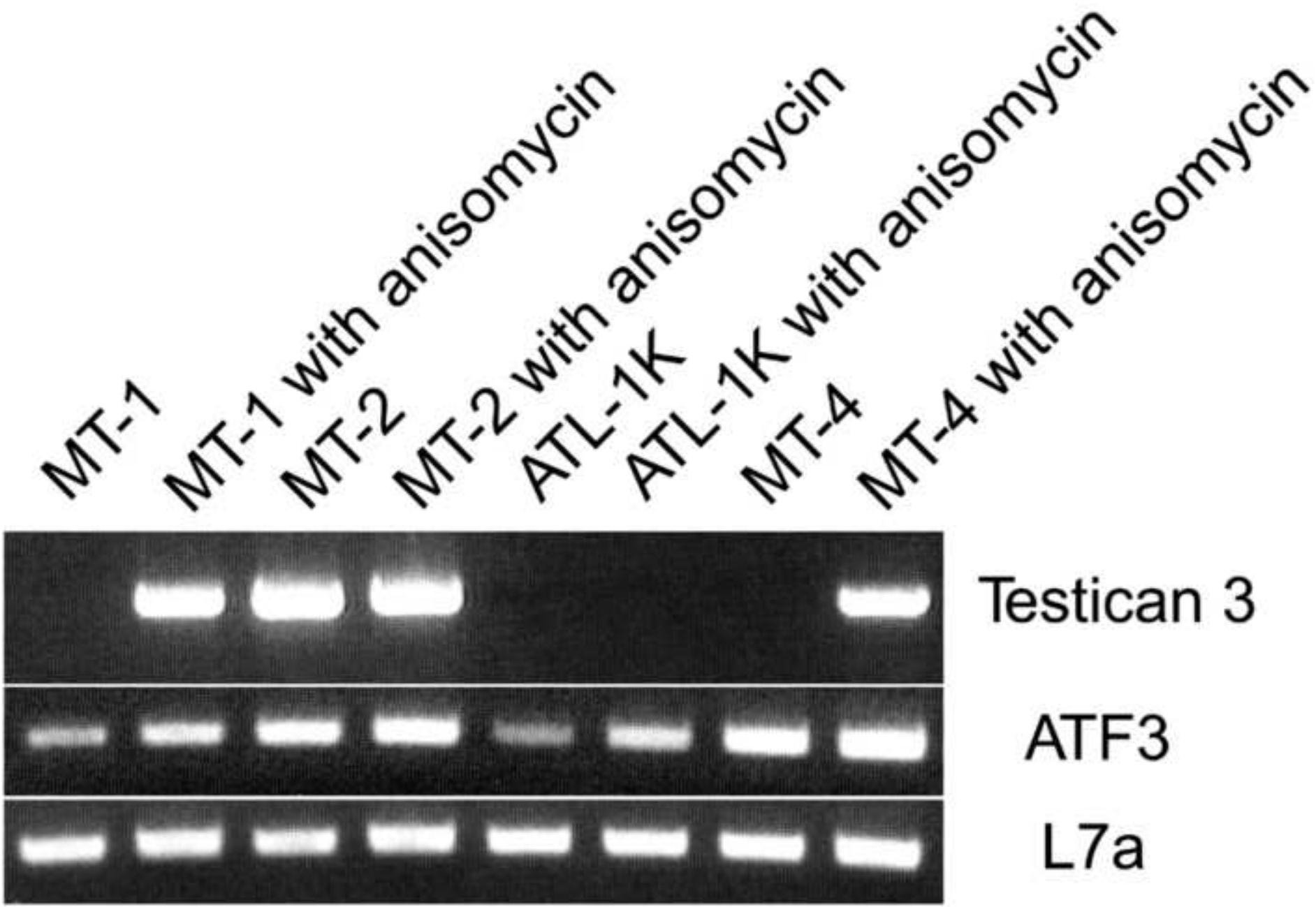

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