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Research Article

RBM10 in complete hydatidiform mole: cytoplasmic occurrence of its 50 kDa polypeptide

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ABSTRACT

Background: RNA-binding motif protein 10 (RBM10), originally identified as S1-1 protein, is a nuclear protein with likely functions in transcription and RNA splicing. The *RBM10* gene maps to the X chromosome and, in female cells, is inactivated in one of the two X chromosomes near the boundary with genes escaping inactivation. This study investigated the occurrence of the *RBM10* gene product in complete hydatidiform mole, which is composed of cells with paternal diploid chromosomes (46, XX).
Methods: Deparaffinized normal chorion or complete hydatidiform mole tissues were hybridized with a fluorescein-conjugated *RBM10* gene probe in fluorescent *in situ* hybridization (FISH) analysis. Immunohistochemistry and immunoelectron microscopy of the tissues were performed using an anti-RBM10 antiserum. Proteins from complete hydatidiform mole tissues and those separated by anti-RBM10-linked affinity chromatography were also examined by western blotting.
Results: As expected, the *RBM10* gene was detected by FISH as double spots in the nuclei of complete hydatidiform mole cells. Immunohistochemistry revealed a nuclear presence of RBM10 in normal chorion and complete hydatidiform moles, and a notable cytoplasmic presence in complete hydatidiform moles. Western blotting and immunoaffinity chromatography revealed that a 50 kDa protein was predominantly found in the cytosolic fraction of complete hydatidiform moles.
Conclusions: A 50 kDa protein with common antigenicity to RBM10 was found in the cytoplasm of complete hydatidiform mole cells, and could represent one of the characteristics of the disease.

Keywords: Complete hydatidiform mole, Cytoplasm, Inactivation, RBM10, S1-1 protein, X chromosome

INTRODUCTION

Hydatidiform mole is a proliferative disease of the chorionic villi, with trophoblastic hyperplasia and cystic enlargement of villus interstitial tissue.[1] Complete hydatidiform mole is usually composed of cells with diploid DNA (46, XX) derived only from paternal chromosomes,[2] while partial hydatidiform mole contains

cells with triploid DNA (69, XXY or 69 XXX) derived from two sets of paternal chromosomes and one set of maternal chromosomes.[3,4]

RNA-binding motif protein 10 (RBM10, originally characterized as S1-1 protein) is a nuclear RNA-binding protein[5] found in splicing complexes,[6,7] and is suggested to function in RNA splicing[8] and transcription.[9] Mutations in *RBM10* are associated with the X-linked disorder TARP syndrome[10] as well as various cancers.[11,12] *RBM10* maps to the X chromosome[8] at the boundary site between inactivated genes and those escaping inactivation.[13] This led us to examine the status of *RBM10* expression in complete hydatidiform mole. We found that RBM10 localized both in the nuclei and cytoplasm of complete hydatidiform mole cells, and that a 50kDa protein with strong antigenicity against anti-RBM10 antiserum was abundantly present in the cytoplasm. The importance of these findings is discussed.

METHODS

FISH of human RBM10 and sex-determining region Y (SRY)

Human DNA was extracted from lymphocytes of male volunteers harvested in Leukoprep tubes (Becton & Dickinson, Franklin Lakes, NJ, USA) using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). A 1,916 bp DNA fragment corresponding to 1,820 bp intronic sequence flanked by 34bp of exon 5 and 62bp of exon 6 of *RBM10* was PCR-amplified using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) with 1mM $MgCl_2$. PCR conditions were 35 cycles of annealing at 60°C for 1 min and polymerization at 72°C for 10 min. Forward and reverse primers were: 5'-GCACGGGAGGTTCGGCTGATG-3' and 5'-CCATCGTGTAGCGTCCTGCAAGTG-3', respectively. A 600 bp DNA fragment of sex-determining region Y (SRY) was PCR-amplified according to the manufacturer's protocol (Maxim Biotech Inc., San Francisco, CA, USA) with the forward primer 5'-GACAATGCAATCATATGCTTCTGC-3' and reverse primer 5'-CTGTAGCGGTCCCGTTGCTGCGGTG-3'. The amplified DNA was labeled with 1 mM biotin-16-dUTP using a Nick Translation Kit (Roche Molecular Biochemicals, Mannheim, Germany) and precipitated with 0.2 μg/ml salmon sperm DNA and 0.2μg/ml *Escherichia coli* tRNA in 80% ethanol at -20°C.

After pathological evaluation, nuclei of hydatidiform moles and artificially aborted normal chorion were isolated from deparaffinized tissues according to Hedley's procedure with modification,[14,15] and pipetted onto slides treated with Vectabond (Vector Laboratories, Burlingame, CA,USA). The nuclei were denatured in 70% formamide in 2×SSC at 70°C for 2 min, then reacted with biotin-16-dUTP-labeled probes at 37°C overnight, and FITC-conjugated avidin at 37°C for 40 min. The slides were washed and mounted with 1 mg/ml *p*-phenylenediamine in buffered-glycerol (pH8.0). Photomicrographs at 1,000× were obtained by fluorescent microscopy using a 470–490 nm excitation filter and a 520–550 nm emission filter.

Immunohistochemistry with anti-RBM10 antiserum

Anti-RBM10 antiserum was raised by immunizing rabbits with a purified recombinant rat RBM10 peptide (amino acids 1–166) produced in *E. coli*.[9] Tissues of complete hydatidiform mole or normal chorion were fixed overnight in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4 μm for pathological evaluation and immunohistochemistry. After deparaffinization, the antigens of sectioned tissues on MAS-coated glass slides were demasked for antigens by autoclaving at 110°C for 20 min in 50 mM citrate buffer (pH 6.0). After inactivation of endogenous peroxidase with 3% H_2O_2, non-specific reactions were blocked with 5% goat serum in phosphate-buffered saline with 0.02% Tween-20, and the tissues were reacted overnight at 4°C with rabbit anti-RBM10 antiserum diluted 1:200, washed and then reacted for 60 min at room temperature with peroxidase-conjugated goat anti-rabbit immunoglobulins. After washing, the antigens were visualized by incubation with 0.1% diaminobenzidine in 0.1 M Tris-HCl buffer and 0.01% H_2O_2.

Electron microscopy with immunogold labeling

After fixation of complete hydatidiform mole or normal chorion tissue sections with Zamboni solution (15% saturated picric acid and 2% paraformaldehyde in phosphate buffer, pH 7.4), the tissues were incubated with 50 mM lysine then incubated for 30 min in phosphate buffer (pH 7.6) containing 5% normal goat serum, 5.8% bovine serum albumin (BSA), 0.25% cold water fish skin gelatin and 0.13% NaN_3. They were then reacted over night at 4°C with rabbit anti-RBM10 antiserum diluted 1:200 with phosphate buffer (pH 7.6) containing 0.8% BSA, 0.25% cold water fish skin gelatin and 0.13% NaN_3, rinsed and reacted with anti-rabbit IgG labeled with ultra small colloidal gold (Aurion, Wageningen, The Netherlands). The tissues were fixed again with 2% glutaraldehyde for 10 min, rinsed with PBS, then postfixed with 1% OsO_4 in PBS. After washing with PBS then with distilled water, they were incubated with Aurion R-Gent enhancer, 50% arabic gum in 0.1 M citric buffer, and developer to visualize the gold particles, then dehydrated before embedding in resin. After staining the sections with uranyl acetate and lead citrate to enhance the contrast, micrographs were taken at 80 kV with a Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan).

Western blotting of cellular proteins

With the informed consent of the patients, tissues of complete hydatidiform mole were homogenized with a

glass Teflon homogenizer on ice in 20 mM Tris-HCl (pH 7.5), 0.005% (w/v) Brij-35, 0.1% (w/v) CHAPS, 10 µg/ml leupeptin hydrochloride, 0.1 mM phenylmethylene sulfonylfluoride. The homogenates were centrifuged at 1,000×*g* for 10 min to remove the nuclear fraction in the pellet. The supernatant was separated by ultracentrifugation at 105,000 × *g* for 1 h to obtain the cytosolic fraction. Proteins of the cytosolic fraction were separated by SDS-PAGE, and stained with Coomassie Brilliant Blue R or blotted on a PVDF membrane (Hybond P, GE Healthcare, Buckinghamshire, UK). The proteins on PVDF membranes were incubated overnight at 4°C with rabbit anti-RBM10 antiserum diluted 1:1,000 then with peroxidase-conjugated goat anti-rabbit immunoglobulins IgG diluted 1:5,000 (ICN Pharmaceuticals, Inc., Aurora, OH, USA), and the immunocomplexes were visualized with ECL-plus immunodetection reagents on Hyperfilm (GE Healthcare).

Affinity chromatography of anti-RBM10 beads

IgGs of rabbit anti-RBM10 antiserum were separated on a Protein A Sepharose column (HiTrap affinity column, GE Healthcare). The IgGs eluted with 0.1 M citric acid (pH 3.0) were then coupled to prepacked HiTrap NHS-activated HP agarose beads (GE Healthcare) to prepare prepacked rabbit anti-RBM10 IgG-agarose beads. The cytosolic fraction of complete hydatidiform mole obtained by ultracentrifugation of the postnuclear fraction was applied to the prepacked rabbit anti-RBM10 IgG-agarose beads and eluted with 0.05% trifluoroacetic acid. Proteins in the eluate were freeze-dried, separated on 10% polyacrylamide SDS gels and stained with Coomassie Brilliant Blue R.

RESULTS

FISH with RBM10 and SRY probes

The *RBM10* gene was detected as single fluorescence spots in the nuclei of artificially aborted normal chorion (Figure 1A), but as double fluorescence spots in the nuclei of complete hydatidiform mole (Figure 1B). By contrast, the *SRY* gene in Y chromosomes was detected as single spots in the nuclei of normal chorion (Figure 1C), but not detected in the nuclei of complete hydatidiform mole (Figure 1D). PCR of normal chorionic DNA with SRY primers confirmed the presence of *SRY* (data not shown).

Immunohistochemistry with anti-RBM10 antiserum

RBM10 was detected using specific anti-RBM 10 antiserum in the nuclei of normal chorion (Figure 2A) and complete hydatidiform mole (Figure 2B). RBM10 immunoreactivity was also found in the cytoplasm of complete hydatidiform mole trophoblast cells, but not in the normal chorion. RBM10 was more prominent in cytotrophoblast cells than in syncytiotrophoblast cells (Figure 2B and 2C).

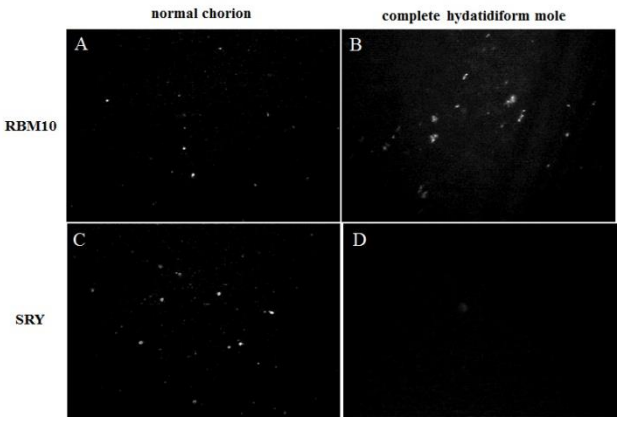

Figure 1: FISH of *RBM10* and *SRY* genes. RBM10 (A, B) or SRY (C, D) DNA probes were labeled with biotin-dUTP by nick translation and reacted with the nuclei of normal chorion (A, C) or complete hydatidiform mole (B, D). FITC-conjugated avidin bound to the DNA probes was detected under a fluorescence microscope.

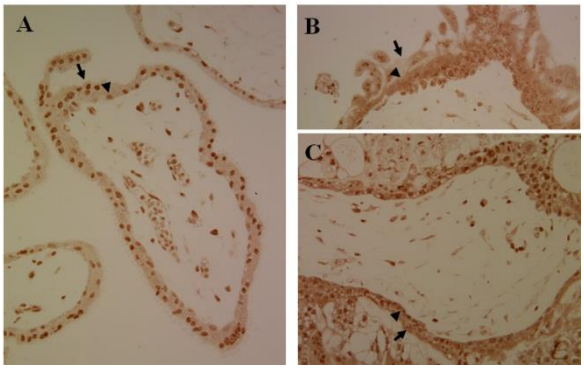

Figure 2: Immunohistochemistry with anti-RBM10 antiserum. Tissues from normal chorion (A) and complete hydatidiform mole (B, C) were deparaffinized. Antigens were demasked by autoclaving and reacted with rabbit anti-RBM10 antiserum then with peroxidase-conjugated anti-rabbit antibody. They were visualized with diaminobenzidine. Arrows and arrowheads indicate cytotrophoblasts (inner layer) and syncytiotrophoblasts (outer layer), respectively.

Electron microscopy using immunogold labeling

Immunogold labeling was observed in the nuclei of normal chorion and complete hydatidiform mole (Figure 3A and 3B). It was also found diffusely distributed in the cytoplasm of complete hydatidiform mole (Figure 3B), and more abundantly in the surface of its intracellular vacuole structures (Figure 3C).

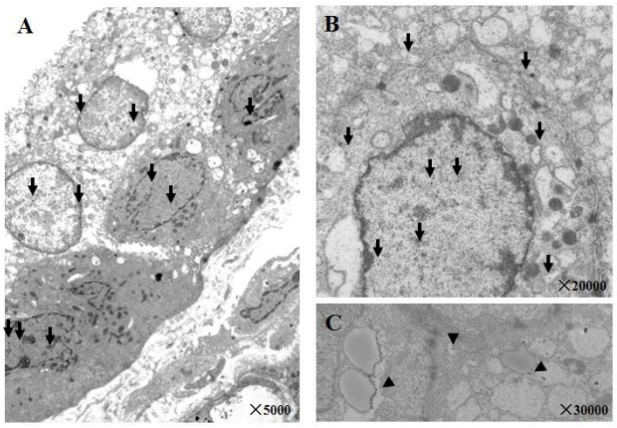

Figure 3: Electron micrograph of immunogold labeling. Tissues of normal chorion (A) and complete hydatidiform mole (B, C) were reacted with rabbit anti-RBM10 antiserum and processed for anti-rabbit IgG immunogold labeling. Arrows show immunogold labeling in the nuclei of normal chorion and in the nuclei and cytoplasm of complete hydatidiform mole. Arrowheads show immunogold labeling in cytoplasmic vacuoles.

Immunoreactive proteins detected with RBM10 antiserum in western blotting

Proteins immunoreactive to anti-RBM10 antiserum with molecular masses of 130 kDa, 58 kDa and 50 kDa were found in the cytosolic fraction of complete hydatidiform mole (Figure 4, lane 3). Only one immunoreactive protein with a molecular mass of 50 kDa was detected in the eluate from the immunoaffinity column linked with anti-RBM IgG (Figure 4, lane 1).

DISCUSSION

Hydatidiform mole is usually composed of cells with diploid chromosomes of paternal origin.[16,17] No report has previously documented hydatidiform moles devoid of X chromosomes, which appear to be essential for mammalian cells. At the same time, an overdose of X chromosome genes in female embryos is compensated for by inactivation of one of the two X chromosomes. Consistent with the presence of an*RBM10* gene on the X chromosome, our FISH analysis involving hybridization with anRBM10 probe revealed two fluorescent dots in a single nucleus in complete hydatidiform mole, which is compatible with the chromosomal composition 46, XX. Homologous chromosomes with identical DNA sequences are the results of chromosome dissociation failure in the first mitosis of the fertilized egg.[3] In contrast to the inactivation of paternal chromosomes in female normal chorion, the inactivation of X chromosome genes rarely occurs in complete hydatidiform moles.

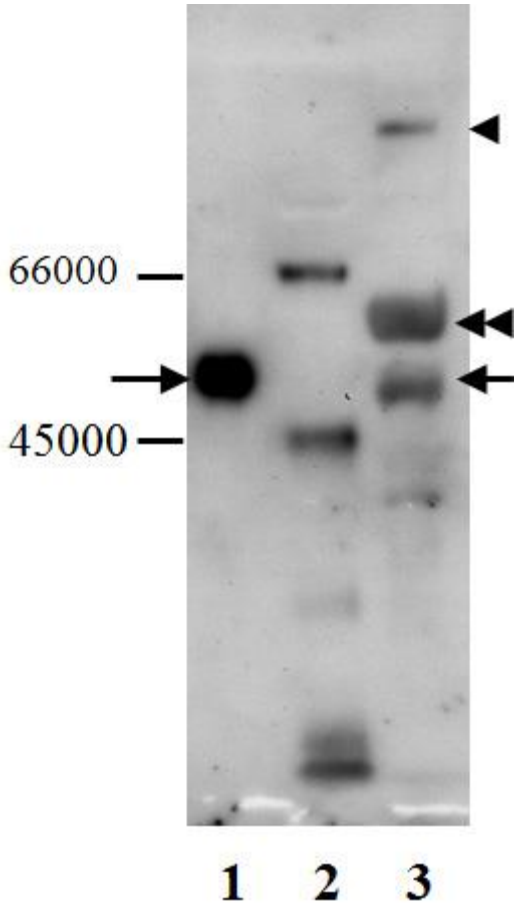

Figure 4: Western blotting with anti-RBM10 antiserum. Proteins of the cytosolic fraction of complete hydatidiform mole (lane 3) and its eluate from the anti-RBM10-linked immunoaffinity column (lane 1) were separated by 10% SDS polyacrylamide gel electrophoresis, blotted onto a PVDF membrane, and reacted with specific rabbit anti-RBM10 antiserum. Molecular weights of standard protein markers (lane 2) are indicated on the left. Arrowhead indicates 130 kDa protein, double arrowheads indicate 58 kDa protein, and arrows indicate 50 kDa protein.

The amino acid sequences of human and rat whole RBM10 proteins share 96.7% similarity, and those of amino acids 1–166 region are 99.5% identical. The antibody used in the present study was raised in a rabbit against a recombinant polypeptide corresponding to amino acids 1–166 of rat RBM10, and thus is highly specific. It usually detects two splicing isoforms in western blotting of nuclear fractions, at 130 and 114 kDa.[9]A 58 kDa protein was previously detected in the human promyelocytic leukemia cell line HL-60, but its corresponding mRNA was not found and the protein was considered to be a proteolytic product of RBM10.[18] The cytoplasmic 50 kDa protein detected in the present study is also considered to be a proteolytic product of RBM10, as the upper 58 kDa band rapidly diminished during prolonged sample-handling time such that the 50 kDa

polypeptide accumulated in the cytoplasmic fraction (data not shown).

Together, our present results indicate that a notable cytoplasmic presence of RBM10 occurs not only in hepatocellular carcinoma cells and cirrhotic hepatocytes,[18] but also in cells of complete hydatidiform mole. This suggests that a cytoplasmic occurrence is a sign or characteristic of disease, involving disruption of normal cell physiology. Further studies are necessary to confirm this.

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REFERENCES

1. Vassilakos P, Riotton G, Kajii T. Hydatidiform mole: Two entities. A morphologic and cytogenetic study with some consideration. Br J Obstet Gynecol 1977;89:167-70.
2. Chew SH, Perlman EJ, Williams R, Kurman RJ, Ronnett BM. Morphology and DNA content analysis in the evaluation of first trimester placentas for partial hydatidiform mole (PHM). Hum Pathol 2000;31:914-24.
3. Kajii T, Ohama K. Androgenic origin of hydatidiform mole. Nature 1977;268:633-4.
4. Lawler SD, Fisher RA, Dent J. A prospective genetic study of complete and partial hydatidiform moles. Am J Obstet Gynecol 1977;127:167-70.
5. Inoue A, Takahashi KP, Kimura M, Watanabe T, Morisawa S. Molecular cloning of a RNA binding protein RBM 10. Nucleic Acid Res 1996;24:2990-7.
6. Rappsilber J, Ryder U, Lammond M. Large-scale proteomic analysis of the human spliceosome. Genome Res 2002;12:1231-45.
7. Hegele A, Kanburov A, Grossmann A, Sourlis C, Wowro S, Weimann M, et al. Dynamic protein-protein interaction wiring of the human spliceosome. Mol Cell 2012;45:567-80.
8. Bonnal S, Martinez C, Forch P, Bachi A, Wilm M, Valcarcel J. RBM5/Luca-15/H37 regulates Fas alternative splice site pairing after exon definition. Mol Cell 2008;32:81-95.
9. Inoue A, Tsugawa K, Tokunaga K, Takahashi KP, Uni S, Kimura M, et al. S1-1 nuclear domains: characterization and dynamics as a function of transcriptional activity. Biol Cell 2008;100:523-35.
10. Gripp KW, Hopkins E, Johnston JJ, Krause C, Dobyns WB, Biesecker LG. Long-term survival in TARP syndrome and confirmation of RBM10 as the disease-causing gene. Am J Med Genet A 2011;155A:2516-20.
11. Furukawa T, Kuboki Y, Tanji E, Yoshida S, Hatori T, Yamamoto M, et al. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. Sci Rep 2011;1:161. DOI: 10.1038/srep00161.
12. Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell 2012;150:1107-20.
13. Coleman MP, Ambrose HJ, Carrel L, Nemeth HF Willard AH, Davies KE. A novel gene, DXS8237E, lies within 20kb upstream of UBE1 in Xp 11.23 and has a different X inactivation status. Genomics 1996;31:135-8.
14. Heiden T, Wang N, Trbukait B. An improved method for preparation of paraffin-embedded tissues for flow cytometric analysis of ploidy and S-phase. Cytometry 1991;12:614-21.
15. Hyytinen E, Vasakorpi T, Kallioniemi A, Kallioniemi OP, Isola JJ. Improved Technique for analysis of formalin-fixed, paraffin-embedded tumors by fluorescence in situ hybridization. Cytometry 1994;16:93-9.
16. Azuma C, Saji F. Application of gene amplification by polymerase chain reaction to genetic analysis of molar mitochondrial DNA. Gynecol Oncol 1991;40:29-33.
17. Trabetti E, Galavotti R, Zanini L, Zardini E, Zatti N, Bernardi F, et al. The parental origin of hydatidiform moles and blightled ova: molecular probing with hypervariable DNA polymorphisms. Mol Cell Probes 1993;7:325-9.
18. Xiao SJ, Wang LY, Kimura M, Kojima H, Kunimoto H, Nishiumi F, et al. S1-1/RBM10: Multiplicity and cooperativity of nuclear localization domains. Biol Cell 2013;105:162-74.

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