Proteomic analysis reveals that MAEL, a component of nuage, interacts with stress granule proteins in cancer cells

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Received September 24, 2013; Accepted October 21, 2013
DOI: 10.3892/or.2013.2836

Abstract. The Maelstrom (MAEL) gene is a cancer-testis (or cancer-germline) gene, which is predominantly expressed in germline cells under normal conditions, but is aberrantly expressed in a range of human cancer cells. In germline cells, MAEL is found predominantly in the nuage, where it plays an essential role in piRNA biogenesis and piRNA-mediated silencing of transposons. However, the role of MAEL in cancer has not been elucidated. We performed immunoprecipitation and Nano-LC-MS/MS analysis to investigate the interactome of MAEL, and identified 14 components of stress granules (SGs) as potential binding partners of MAEL in MDA-MB-231 human breast cancer and SW480 colorectal cancer cells. The interactions between MAEL and 8 of these SG components (PABPC1, YBX1, KHSRP, SYNCRIP, DDX39, ELAV1, EIF4A1 and EIF3F) were confirmed by anti-tag immunoprecipitation. Immunofluorescence analysis showed that MAEL co-localizes with the SG marker PABPC1 in SGs during oxidative stress. Nuages and SGs are the cytoplasmic RNA granules of germline cells and stressed somatic cells, respectively, and both serve as a platform for small RNA-mediated gene silencing. It is, therefore, suggested that MAEL may be involved in miRNA-mediated gene silencing in SGs, as it does in the nuage. This finding should be valuable toward understanding the function of MAEL in carcinogenesis.

Introduction

The Maelstrom (MAEL) gene was first identified in Drosophila (1). It plays a role in the establishment of oocyte polarity by acting in the positioning of mRNA and the microtubule-organizing center (MTOC) in early oocytes (1,2). Drosophila maelstrom protein co-localizes with Vasa and Aubergine in the nuage (3), a germline-unique perinuclear structure that serves as a platform for PIWI-interacting RNA (piRNA) biogenesis and piRNA-dependent silencing of transposons and some other harmful selfish elements (4,5). The mouse homolog of MAEL is also found to localize in the nuage (6,7) and is essential for spermatogenesis and transposon repression (7). Further study by Aravin et al (8) demonstrated that MAEL co-localizes with MIWI2/PIWIL4 in a type of nuage named piP-body, which harbors both piRNA pathway proteins (MIWI2, TDRD9 and MAEL) and P-body (processing body) components (DDX6, DCP1a, XRN1 and GW182), and that loss of MAEL disrupts normal MIWI2 localization and piRNA production leading to transposon activation. In rat spermatogenic cells, MAEL protein is detected in all types of nuage except the cluster of 30-nm particles (i.e. 70- to 90-nm particles, satellite body, intermitochondrial cement, cluster of 60- to 90-nm particles, chromatoid body) (9). MAEL protein is also found in non-nuage compartments (10) and the nucleus (3,9). In the nucleus, MAEL binds the miR-7 promoter and represses its expression (11).

Our previous study demonstrated that MAEL is a cancer-testis (CT) gene (12). The CT genes, also known as cancer-germline (CG) genes, are predominantly expressed in germ cells of the testis or and ovary and have no or little expression in somatic adult tissues, but are aberrantly expressed in various types of cancers (13,14). We found that the human MAEL gene is only expressed in germ cells of the testis among normal tissues, but is expressed in various human cancer cell lines such as breast cancer MDA-MB-231 cells and colorectal cancer SW480 cells. Like most of the CT genes (15,16), the expression of the MAEL gene is activated by hypomethylation in cancer (12). Kim et al (17) analyzed the methylation profile of 27,578 CpG sites spanning >14,000 genes in colorectal cancer and adjacent normal mucosa, and found that among the genes tested, MAEL and SFT2D3

Key words: MAEL, cancer-testis gene, nuage, stress granule, proteomic analysis
showed the lowest methylation level in tumor tissues when compared to the normal mucosa (17). However, the precise subcellular localization and function of MAEL in cancer cells are not clear.

In the present study, we identified the interacting partners of MAEL in breast cancer MDA-MB-231 and colorectal cancer SW480 cells using mass spectrometric method, and demonstrated that MAEL localizes in stress granules (SGs) in cancer cells and interacts with several components of SGs.

Materials and methods

Plasmids. The Myc-tagged YBX1 expression plasmid pDESTmycYBX1 [Addgene plasmid 19878, deposited by Landthaler (18)] was obtained from Addgene (Cambridge, MA, USA). The other expression plasmids (pHA-MAEL, pMyc-DDX39, pMyc-DDX39, pMyc-EIF4A1, pMyc-EIF3F, pMyc-ELAV1, pMyc-EIF3F, pMyc-ELAV1, pMyc-PABPC1, pMyc-SYNCRIP and pMyc-KHSRP) were constructed as follows. The coding sequence of each gene was amplified from the first strand cDNA of MDA-MB-231 or SW480 cells with the primers described in Table I. The amplified products were cloned into the pMD18-T vector (Takara, Dalian, China) by TA cloning method, and subcloned into the pCMV-HA or pCMV-Myc vector (Clontech Laboratories, Inc., Mountain View, CA, USA) with restriction enzyme sites in the primer (Table I).

Immunoprecipitation (IP) of the MAEL protein complex. The human breast cancer MDA-MB-231 and colorectal cancer SW480 cells [American Tissue Culture Collection (ATCC), Manassas, VA, USA] were cultured in L-15 media that was supplemented with glutamine, antibiotics and 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. The cells were washed with phosphate-buffered saline (PBS) and incubated with 0.5 mM cross-linker DSP (Pierce, Rockford, IL, USA) for 30 min. After cross-linking, cells were sequentially washed with PBS, PBS containing 25 mM Tris-HCl buffer and PBS. Then, cells were lysed in RIPA buffer by sonication, and subjected to centrifugation at 12,000 x g for 15 min. The supernatants were transferred to a new tube for immunoprecipitation.

Immunoprecipitation was performed as described by Lin et al (19). In each 1.5-ml tube, 2.5 mg of lysates was mixed with 2 µg of polyclonal antibody against MAEL or preimmune rabbit IgG (Signalway Antibody Co., Ltd., Nanjing, China), and incubated overnight at 4°C with gentle shaking. Immune complexes were collected on protein A/G agarose beads and washed three times to remove non-specific binding using the same buffer as for the cell lyses. Proteins were eluted from the beads by boiling the beads in loading buffer for 10 min, and were then separated on SDS-PAGE gel and visualized with silver staining.

Nano-LC-MS/MS. Each excised protein gel band was destained and digested with trypsin as described by Lin et al (19). Nano-LC MS/MS experiment was performed on an HPLC system composed of two LC-20AD nano-flow LC pumps, an SIL-20AC autosampler and an LC-20AB micro-flow LC pump (Shimadzu, Tokyo, Japan) connected to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Sample was loaded on a CapTrap column (0.5 x 2 mm; Michrom Bioresources, Inc., Auburn, CA, USA) for 6 min at a flow rate of 25 µl/min. The sample was subsequently separated by a C18 reverse-phase column (0.10 x 150 mm, packed with 3 µm Magic C18AQ particles; Michrom Bioresources) at a flow rate of 500 nl/min. The mobile phases were 2% acetonitrile with 0.1% formic acid (phase A and the loading phase) and 95% acetonitrile with 0.1% formic acid (phase B). To achieve proper separation, a 60-min linear gradient from 0 to 80% phase B was employed. The separated sample was introduced into the mass spectrometer via an Advance 30-µm silica tip (Michrom Bioresources). The spray voltage was set at 1.6 kV and the heated capillary at 180°C. The mass

Table I. Primers used for amplifying the coding region of the genes.

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<td>MAEL-R</td>
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<td>KHSRP-R</td>
<td>GGTACCGATTCAATGAGGCTCGG</td>
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spectrometer was operated in the data-dependent mode, and each cycle of duty consisted of one full-MS survey scan at the mass range 385-2000 Da with resolution power of 100,000 using the Orbitrap section, followed by MS/MS experiments for 10 strongest peaks using the LTQ section. The AGC expectation during full-MS and MS/MS were 1,000,000 and 10,000, respectively. Peptides were fragmented in the LTQ section using collision-induced dissociation with helium and the normalized collision energy value set at 35%. Previously fragmented peptides were excluded for 60 sec.

**Database search.** Tandem mass spectra were extracted by BioWorks version 3.3.1 SP1 (Thermo Fisher Scientific). All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific; version 28). Sequest was set up to search the human proteome database from UniProt release 2010_08 (downloaded from the official website of UniProtKB following this link: http://www.uniprot.org/uniprot/?query=organism:9606+keyword:181&format=*&compress=yes, downloaded at 2010-07-13) assuming the digestion enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 20 ppm. Oxidation of methionine (+15.99492 Da) and acetylation of lysine (+42.01057 Da) were specified as variable modifications. Trans-Proteomic Pipeline (20) was used to validate MS/MS based peptide and protein identifications. Peptide probability was specified by the Protein Prophet algorithm (21). Protein probabilities were assigned by the Protein Prophet algorithm (22). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

**Gene Ontology analysis.** Gene Ontology (GO) enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool (http://david.abcc.ncifcrf.gov) (23). UniProt accession numbers of MAEL-associated proteins were submitted to DAVID system and categorized based on biological process (BP), molecular function (MF) and cellular component (CC) using the software.

**Anti-tag co-immunoprecipitation.** Transfection and co-IP were performed as previously described (24). Hek293 cells were co-transfected with HA-tagged MAEL expression plasmids and expression plasmids of each Myc-tagged SG gene. At 24 h after transfection, cell lysates were prepared and immunoprecipitated with rabbit anti-HA polyclonal antibody or preimmune rabbit IgG, and the precipitated protein was detected by western blot analysis using the anti-Myc monoclonal antibody. The anti-HA, anti-Myc and IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Immunofluorescence staining of SGs.** SW480 cells were grown on glass coverslips, and treated with 0.5 mM hydrogen peroxide (H₂O₂) for 1 h to induce the formation of SGs. Treated and control cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100, respectively. To reduce the unspecific binding, the cells were incubated in 1% BSA overnight the 4°C. Then, cells were incubated with anti-MAEL polyclonal antibody and anti-PABPC1 monoclonal antibody (Pierce, Rockford, IL, USA) for 2 h at room temperature, followed by incubation with Texas Red-conjugated anti-rabbit IgG (red) and FITC-conjugated anti-mouse IgG (green) for 1 h. The nuclei were labeled by DAPI. Images were obtained using a Zeiss LSM 510 confocal microscope (Carl Zeiss Imaging, Oberkochen, Germany). Texas Red-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology.

**Results**

**RNA-binding proteins are enriched in the MAEL protein complex.** To identify the potential interacting partners of MAEL protein, we performed the co-IP experiments to isolate the MAEL complex in human breast cancer MDA-MB-231 and colorectal cancer SW480 cells. The immune complexes of the anti-MAEL antibody and IgG were resolved on SDS-PAGE gel followed by silver staining. The protein bands which only existed in the complex of the anti-MAEL antibody are indicated by arrows.

![Figure 1. Silver-stained SDS-PAGE gel of the immunoprecipitates. The lysates (2.5 mg) of (A) MDA-MB-231 or (B) SW480 cells were mixed with 2 µg of anti-MAEL antibody or IgG, and incubated overnight at 4°C with gentle shaking. Immune complexes were separated on SDS-PAGE gel and visualized with silver stain. The protein bands which only existed in the complexes of the anti-MAEL antibody are indicated by arrows.](http://www.oncology-reports.com/31/3/342-350/2014/image304x562.png)
In the cellular component (CC) category, the term with the lowest P-value was the non-membrane-bound organelle, and that with the second lowest P-value was the ribonucleoprotein complex term. Additionally, a large number of MAEL-associated proteins were enriched in the nuclear lumen, consisting of 23 proteins (29.5% of the 70 proteins assigned to CC category). In the biological process (BP) category, the top three terms with the lowest P-value were RNA process, chromatin assembly and macromolecular complex assembly. When considering all three categories, we found that most MAEL-associated proteins were RNA-binding proteins existing in the ribonucleoprotein complex.

**MAEL interacts with stress granule proteins.** MAEL protein is a component of nuage (3,6,7), a germline-unique ribonucleoprotein complex particle. According to the above results (Table II), MAEL associates with RNA-binding proteins, possibly a component of the ribonucleoprotein complex in cancer cells. However, we did not find other nuage components in the MAEL-associated proteins. Notably, the MAEL-associated proteins contained 14 components of SGs: PABPC1 (25), FUBP1/FBP1 (26), KHSRP/FBP2 (26), YBX1/YB-1 (27), SYNRIP/hnRNP Q (28), EIF3F (29), EIF4A1 (30), EIF4B (31), ELAVL1 (32), HNRNPA1 (33), HNRNPA2B1 (34), DDX1 (27), DDX3X (35) and DDX39 (36). Among these, 8 proteins (PABPC1, FUBP1, KHSRP, YBX1, SYNRIP, EIF4B, HNRNPA1 and HNRNPA2B1) were identified from both MDA-MB-231 and SW480 cells (Table III); 6 proteins were found only in MDA-MB-231 (EIF4A1, EIF3F and DDX3X) or SW480 cells (DDX39, ELAVL1 and DDX1) (data not shown).

**Table II. Top 5 significantly enriched GO terms in the MAEL-associated proteins.**

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<th>Percentage</th>
<th>Value</th>
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<td>RNA binding</td>
<td>25</td>
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<td>Structural constituent of the cytoskeleton</td>
<td>10</td>
<td>12.8</td>
<td>4.04E-10</td>
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<td>Structural molecule activity</td>
<td>15</td>
<td>19.2</td>
<td>1.57E-05</td>
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<td>Transcription corepressor activity</td>
<td>6</td>
<td>7.7</td>
<td>1.60E-03</td>
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<tr>
<td></td>
<td>NAD or NADH binding</td>
<td>4</td>
<td>5.1</td>
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<tr>
<td>CC</td>
<td>Non-membrane-bound organelle</td>
<td>38</td>
<td>48.7</td>
<td>8.98E-10</td>
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<td>Ribonucleoprotein complex</td>
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<td>23.1</td>
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<td>Spliceosome</td>
<td>11</td>
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<td>Intermediate filament</td>
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<td>Nuclear lumen</td>
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<td>RNA splicing</td>
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<td>Chromatin assembly</td>
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<td>Macromolecular complex assembly</td>
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<td>Ectoderm development</td>
<td>8</td>
<td>10.3</td>
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<td>Intermediate filament-based process</td>
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MF, molecular function; CC, cellular component; BP, biological process.
Then, 8 (PABPC1, KHSRP, YBX1, SYNCRIP, EIF4A1, DDX39, ELAVL1 and EIF3F) of the identified SG proteins were selected for further confirmation of their interactions with MAEL protein by co-IP experiments. The coding regions of these 8 genes were cloned into mammalian expression vector pCMV-Myc for constructing Myc-tagged expression plasmids. The Myc-tagged expression plasmid of each SG protein was co-transfected with HA-tagged MAEL expression plasmid into Hek293 cells, respectively. The anti-tag immunoprecipitation assays showed that all these 8 SG proteins were able to interact with the MAEL protein (Fig. 2).

**MAEL localizes to SGs.** As stated above, MAEL protein interacts with SG proteins. Therefore, we performed immunofluorescence analysis to investigate whether the MAEL protein localizes in SGs. In the untreated SW480 cells, MAEL proteins were distributed homogeneously in both the cytoplasm and the nucleus (Fig. 3). After induction with H$_2$O$_2$, some cytoplasmic MAEL proteins displayed a distribution pattern of distinct speckles, and co-localization with SG marker PABPC1 (25) in the speckle (Fig. 3), suggesting that MAEL proteins localize to SGs. Strangely, after treatment with H$_2$O$_2$, PABPC1 did not condense into apparent speckles as it did in the cells treated with arsenite (37).

**Discussion**

Our previous study demonstrated that the human MAEL gene is only expressed in the spermatocytes and spermatids of the testis under normal condition, but is aberrantly expressed in various types of human cancer cells (12). The role of MAEL in spermatogenesis has been studied thoroughly, but no investigation on its function in cancer has been performed. In the present study, we identified 178 MAEL-associated proteins in breast cancer MDA-MB-231 cells and 167 in colorectal cancer SW480 cells by immunoprecipitation and Nano-LC-MS/MS analysis. In these MAEL-associated proteins, we found 14 components of SGs, but none of the nuage. The protein interactions between MAEL and these stress granule proteins were confirmed by anti-tag immunoprecipitation. Immunofluorescence analysis showed that MAEL co-localizes with PABPC1 in SGs during oxidative stress. This is not surprising, given that both SGs and nuage are cytoplasmic ribonucleoprotein complex particles (RNA granules). Nuage is a germline-unique structure (4), whereas SGs are somatic RNA granules and are assembled when cells are exposed to stress (25,38,39). However, both nuage and SGs have similar composition, and serve as sites for small RNA-mediated gene silencing (40). MAEL has been found predominantly in the nuage of germline cells, where it plays an indispensable role in piRNA biogenesis and piRNA-mediated silencing of transposons (3,6,7). Therefore, we hypothesized that MAEL plays a role in miRNA-mediated gene silencing in SGs, as it does in the nuage (7).

Additionally, we demonstrated that in cancer cells, the MAEL protein is distributed in both the cytoplasm and the nucleus, similar to its localization in germline cells (3,7). In the nucleus, MAEL functions as a regulator of gene expression. For example, mouse MAEL was found to interact with chromatin-remodeling factors SNF5 and SIN3B in the testis (6); *Drosophila* MAEL represses the transcription of miR-7 (11). In accordance with the nuclear function of MAEL in germline cells, Gene Ontology annotation showed that some MAEL-associated proteins in cancer cells are involved in chromatin remodeling and transcription repression.

Our results suggest that MAEL plays a similar role in germline and cancer cells, similar to most CT genes. For example, GAGE functions as a regulator of chromatin reorganization in both germ and cancer cells (41). To date, 156 families of CT genes have been collected in the CT database (http://www.cta.lncc.br). The discovery of CT genes led to the hypothesis that gametogenesis and carcinogenesis share a similar mechanism; the expression of germine genes in cancer reflects the activation of the silenced gametogen-
Table III. The SG proteins identified in the MAEL complexes from both MDA-MB-231 and SW480 cells.

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<th>Accession no.</th>
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esis programme in somatic cells; this programme might contribute characteristic features to the neoplastic phenotype, including immortality, invasiveness, hypomethylation and metastatic capacity (42). Costa et al (43) believed that CT genes play a role in stem cell self-renewal; the expression of CT genes in tumor tissues contributes to maintaining stem cell properties and favors tumor proliferation. This hypothesis was supported by Yamada et al (44) who found that considerable numbers of CT genes showed preferential expression in cancer stem-like cells. Previous studies have shown that MAEL is necessary for proper germline stem cell lineage differentiation (11) and knockdown of MAEL expression disrupts the differentiation of mouse embryonic stem cells into germ cells (45). Therefore, whether MAEL also plays a role in cancer stem cells warrants further investigation.

In summary, we used proteomic approaches for isolating the interacting partners of MAEL, and demonstrated that MAEL, a component of nuage in germline cells, localizes in SGs and interacts with several components of SGs, suggesting that MAEL plays a role in carcinogenesis by post-transcriptionally regulating gene expression, as it does in gametogenesis. This finding should be valuable toward understanding the function of MAEL in carcinogenesis.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81272318 and 81071656).

References


