S100A9 is a novel ligand of EMMPRIN that promotes melanoma metastasis

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Supplementary data (online)

Supplementary Materials and Methods

Preparation of expression vectors. Briefly, we first prepared two mammalian expression vectors by modifying the promoter less pDNR-1r (Clontech TAKARA, Mountain View, CA) and pIDT-SMART (Integrated Device Technology, San Jose, CA) vectors using CMV promoter-intron (CMVi) from the phCMV-FSRTM vector (Genlantis, San Diego, CA) and the CMVi with a part of the HTLV type 1 LTR (RU5’ ) to express even shorter cargo cDNAs at very high levels (13). We designated the former, pDNR-CMVi and the latter, pIDT-CMViR, respectively. The human cDNAs encoding extracellular domain of EMMPRIN [soluble (sol) EMMPRIN: 1-205 aa] and cytoplasmic domain deficient EMMPRIN [dominant negative (dn) EMMPRIN: 1-231 aa] were inserted to the pDNR-CMVi vector. The solEMMPRIN and dnEMMPRIN were designed to express Myc-HA-Flag-6His-tagged and 6His-tagged forms at the C-terminal ends, respectively (pDNR-CMVi-solEmmp. and –dnEmmp.). The short cytoplasmic tail of a RAGE variant [RAGE-cyt (S391E: phospho-mimic form), 364-404 aa] (13) and EMMPRIN variants [Emmp.-cyts (wt, mut-traf: P235G+V238G+D240G+D241G, and mut-C ter.: K259G+K261G+R264G+R266G), 230-269 aa] were cloned into pIDT-CMViR vector [pIDT-CMViR-RAGE-cyt (S391E) and -Emmp.-cyts (wt, mut-traf, and mut-C ter.)]. These cytoplasmic domains were constructed to express N-terminal (RAGE-cyt) and C-terminal (Emmp.-cyt) 6His-2HA tagged forms, respectively. The adaptors, TIRAP, MyD88, TRAF2, TRAF6, dnTRAF2 (86-501 aa) and dnTRAF6 (289-522 aa) (21) were tagged with Myc at the C-terminal end and cloned into pIDT-CMViR vector. Human cDNAs encoding S100A8 and S100A9 were also tagged with N-terminal HA and C-terminal 6His, and
they were cloned into the pEF6/myc/his vector (pEF-S100A8 and –S100A9; Invitrogen, Carlsbad, CA).

**Keratinocyte culture.** Human keratinocytes derived from normal foreskin were obtained from Krabou and cultured in Humedia KG2 (Kurabo, Osaka, Japan).

**List of PCR primers**

**List of primary antibodies**
Primary antibodies used were, anti-S100A9 antibody (Calgranulin B, sc-8114), anti-collagen, type VII (COL7A1, H-120l; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-human CD147 (BioLegend, San Diego, CA), anti-melanoma antibody (HMB-45; Gene Tex, Inc) and anti-Melan A/MART-1 antibody (ANASPEC, Inc., San Jose, CA). Anti-mouse CD31 was from BD Bioscience (Franklin Lakes, NJ) and monoclonal anti-actin, α-smooth muscle-Cy3™ antibody (Sigma-Aldrich, St. Louis, MO) were used for the analysis of blood vessels.

**Proximity Ligation Assay (PLA)**
PLA reaction was performed using the each two antibody combinations from monoclonal anti-EMMPRIN antibody and anti-S100A9 antibody for the sections of melanoma tissues, according to the manufacture’s instructions. Antibody dilutions used were; 0.5 μg/ml and 1 μg/ml, respectively.