Expression of MS4A and TMEM176 genes in human B lymphocytes

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INTRODUCTION

MS4A is a family of tetraspanning membrane proteins, most of which remain uncharacterized (1, 2). Phylogenetic analysis indicated that the genes encoding these proteins first appeared and expanded in early vertebrates, with rapid diversification such that homologs of some of the human MS4A genes are not found in the mouse and vice versa (3). The founding member of this family, MS4A1 (CD20), is expressed at high levels in B-lymphocytes, is associated with the B-cell antigen receptor (BCR) (4), functions in BCR-activated calcium influx (5), and is required for optimal humoral immunity (6). Expression of CD20 first occurs during the pre-B stage of development and is lost upon terminal differentiation to plasma cells. CD20 is also expressed at high levels in B-cell lymphomas, and is an effective target for therapeutic depletion of pathogenic B-cells in various disease settings (7–9). Rituximab and other CD20-directed antibody therapeutics bind to epitopes on one or both of the two small extracellular loops between the transmembrane spanning regions (10–13). The short length of these loops brings bound antibodies close to the plasma membrane, a property that is likely to contribute to the efficacy of CD20 as a target (9). Antibody-mediated stabilization of CD20 in lipid rafts (14, 15) appears to allow enhanced complement activation (16), and the slow rate of internalization of antibody-bound CD20 allows prolonged display of Fc regions to Fc receptor-bearing effector cells (17–19). Some or all of these factors are likely to be shared by other MS4A proteins, which could thus be a source of potentially novel immunotherapeutic targets.

CD20 exists as homo-oligomers in the plasma membrane (4, 20), however it has been suggested that different MS4A proteins expressed in the same cell may hetero-oligomerize with one another. Indeed, hetero-oligomerization of murine MS4a4B and MS4a6B has been reported (21), and unusual MS4A genes with...
fused repeating MS4A sequences occur in some species (3), suggesting that hetero-oligomerization may be a general feature of MS4A proteins. Given the importance of human CD20 to BCR signaling, humoral immunity, and immunotherapeutic depletion of B-cells, and the potential for other MS4A proteins to hetero-oligomerize with CD20, we sought to identify non-CD20 MS4A genes expressed in primary human B-cells. Most of the known MS4A genes have expression that is restricted to other tissues, particularly myeloid-lineage cells, testes, lung, andintestinal tissues; however, MS4A4A, MS4A6A, MS4A7, and MS4A8B were reported to be expressed in human B-cell lines (2). We investigated the quantitative expression of these 4 MS4A genes, as well as two genes comprising the closely related TMEM176 gene family (3), in human blood and tonsil B-cells with a view to identifying candidates for future investigation at the protein level. A secondary goal was to identify MS4A proteins that might provide alternate therapeutic targets for chronic lymphocytic leukemia (CLL), a B-cell malignancy in which CD20 is expressed at muchlower levels than in B-cell lymphomas (22–24). We therefore also investigated expression of MS4A and TMEM176 genes in a group of untreated CLL patients.

MATERIALS AND METHODS

CELL ISOLATION

All samples in this study were obtained by approval of the Conjoint Health Research Ethics Board at the University of Calgary. Blood samples from healthy volunteer donors were obtained from laboratory personnel in the Immunology Research Group, University of Calgary. Tonsils were obtained from patients undergoing tonsillectomy at the Alberta Children’s Hospital. Whole blood from patients with CLL was obtained anonymously as leftover samples from the Flow Cytometry Laboratory of Calgary Laboratory Services at Foothills Medical Center; only samples with dim CD20 expression and ≥96% CD5+ B-cells were included in the study. All samples were processed within 24 h of collection. B-cells were purified from whole blood using the RosetteSep™ Human B-cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC, Canada) and from tonsils using a modified RosetteSep™ method (25). CD5+ B-cells were prepared from normal blood B-cells using the EasySep® Positive Selection Kit (StemCell Technologies) with PE-conjugated anti-CD5 (BioLegend, Mississauga, ON, Canada). CD27 positive selection was achieved using CD27-PE with the EasySep® Positive Selection kit (StemCell Technologies) with the nearest neighbor algorithm, Microtome for Windows (West Grove, PA, USA). Digital deconvolution was performed by confirming the expected sizes and sequences of the amplified sequences. Amplification of specific products was further validated during PCR optimization by gel purification and sequencing. Amplification efficiencies, calculated using 10-fold dilutions (10⁻¹ to 10⁻²) of cDNA prepared from tonsil B-cell RNA, were close to 2.0 in every case (Table 1). Data were normalized to an endogenous control, ACTB (β-actin). mRNA levels in each sample were thus expressed as the difference between threshold values (ACT) for the gene transcript of interest and ACTB, using the equation Ratio = (Eref=target(ΔCt−ΔCt)/Eref=control−sample((ΔCt−ΔCt=sample)). (26).

cDNA CONSTRUCTS AND TRANSFECTIONS

The human MS4A4A, MS4A6A, MS4A7, and MS4A8B coding sequences were cloned and inserted into the multicloning site (MCS) of the pEGFP-C1 vector (Clontech, Mountain View, CA, USA). BJAB cells were electroporated at 340 V and 950 µF (Gene Pulser II, Bio-Rad, Hercules, CA, USA) with 20 µg of cDNA. GFP-positive cell lines were generated in 1 mg/ml Geneticin (Life Technologies). BJAB cells stably expressing GFP-CD20 were previously established (13).

IMMUNOFLUORESCENCE MICROSCOPY

Cells were incubated with antibodies either before or after fixation in 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA, USA) at room temperature for 15 min. Antibodies against EEA1, golgin 95, golgin 97, and giatin were provided by Dr. Marvin Fritzler (University of Calgary, AB, Canada). CD20 was provided by Dr. J. Ledbetter (Bristol-Myers Squibb, Seattle, WA, USA). Cy3-conjugated F(ab′)2 goat anti-mouse IgG was purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). Nuclei were stained with 4′,6-Diamidino-2-phenylindole (DAPI, Sigma). Fluorescence imaging was done with a Leica DM RXA microscope (Rochester, NY, USA) attached to a 14-bit cooled CCD camera (Princeton Instruments, Monmouth Junction, NJ, USA). Digital deconvolution was performed using the nearest neighbor algorithm, Microtome for Windows (Vaytek, Fairfield, IA, USA).

RESULTS

The MS4A4A, MS4A6A, MS4A7, and MS4A8B genes were selected for analysis in this study on the basis of evidence that they are expressed in B-cell lines (2). MS4A genes with expression known to be limited to myeloid-lineage cells or non-hematopoietic tissues were excluded. MS4A1 (CD20), which is expressed at high levels in mature B-cells, was included for comparison. In addition, we examined expression of the MS4A-related genes, TMEM176A and TMEM176B, which have not previously been assessed in human B-cells. The levels of expression were assessed using quantitative RT-PCR (qPCR), with validation of the primer sets done by confirming the expected sizes and sequences of the amplified products. Amplification of each gene was compared to the that of ACTB (β-actin) and shown on the y-axes in Figures 1 and 2 as Relative mRNA.
Table 1 | Human MS4A/TMEM176 qPCR primer sequences and properties.

| Primer name  | Accession number | Sequence                          | Melting temp. | AE \(^1\) |
|--------------|------------------|-----------------------------------|---------------|-----------|
| qhMS4A1F     | NM_152866.2      | CAC CCA TCT GTG TGA CTG TGT G     | 68            | 1.98      |
| qhMS4A1R     |                 | AGT TTT TCT CCG TTG CTG CC        | 60            |           |
| qhMS4A4AF    | NM_148975.2      | TGG CTG TCA TAC ATT CAC ATC TG     | 60            | 2.04      |
| qhMS4A4AR    |                 | CCA TAC ACA TCA TTG TTA TCC CCA   | 60            |           |
| qhMS4A6AF    | NM_152852.2      | CAC GCA GAA ATC AAA GTT ATT G      | 60            | 1.98      |
| qhMS4A6AR    |                 | TGG GTA AGC AGA GTT CAA CAG TG     | 68            |           |
| qhMS4A7F     | NM_021201.4      | CAC CAA AGG GCA TCA CTA TCC       | 64            | 1.92      |
| qhMS4A7R     |                 | GAA ATC AAC AGG CAA CAC AGG        | 62            |           |
| qhMS4A8BF    | NM_031457.1      | GAT CTC TCT CCG TGG CAG C          | 60            | 2.19      |
| qhMS4A8BR    |                 | TGA CGA TGT TCA AGC CCA AAC        | 60            |           |
| qhTMEM176AF  | NM_018487.2      | GAG TCC AGA AGA AGT CAG AAG GC     | 70            | 2.06      |
| qhTMEM176AR  |                 | AAG CAG CAG AAT CCA GAC ACC        | 64            |           |
| qhTMEM176BF  | NM_014020.3      | GGC AGA AGA AGG AGT GTA GAG C      | 70            | 1.98      |
| qhTMEM176BR  |                 | CAG GAA CAG GGC AGC GAT T          | 60            |           |
| qhB-ACTINR   | NM_001101.3      | GTG TTG GCG TAC AGG TCT TTG        | 64            | 2.01      |
| qhB-ACTINF   |                 | CAC TCT TCC AGC CTT CCT TCC        | 66            |           |

\(^1\) Amplification efficiency.

FIGURE 1 | MS4A/TMEM176 mRNA levels in non-hematopoietic human tissues. cDNA was prepared from tissue RNA samples in the human total RNA master panel II (Clontech). Quantitative PCR was performed to amplify MS4A1, MS4A4A, MS4A6A, MS4A7, MS4A8B, TMEM176A, TMEM176B, and ACTB cDNA using primer sets with equivalent amplification efficiencies (Table 1). Vertical axes show mRNA levels relative to ACTB.

We first examined the expression of the MS4A and TMEM176 genes in non-hematopoietic tissues (Figure 1). Detection of MS4A1 (CD20) transcripts in the lung, trachea, thyroid, and uterus is probably indicative of blood contamination or infiltration of these tissues. Among non-hematopoietic tissues, MS4A4A detection was limited to the lung. Considering that MS4A1 (CD20) transcripts were also amplified from the lung, MS4A4A may be expressed on a hematopoietic cell type in that tissue. MS4A8B was expressed most highly in the lung and trachea, consistent with a previous report (27), and was also expressed in a few other tissues but with a more restricted profile than the remaining genes, MS4A6A, MS4A7, TMEM176A, and TMEM176B, which were detected broadly in non-hematopoietic tissues (Figure 1).
We then examined expression of the MS4A4A and TMEM176 genes in B-cells isolated from multiple donor samples of peripheral blood and tonsil tissues. As expected, MS4A1 (CD20) transcripts were always detected at high levels (Figure 2A). Surprisingly, however, we detected only relatively low levels of transcripts for any of the six new MS4A/TMEM176 genes in either tonsil or peripheral blood B-cells. Indeed, MS4A4A and MS4A8B transcripts were not detected in any sample (Figure 2A). MS4A6A and MS4A7 transcripts were detected in most B-cell samples but at two to four orders of magnitude lower than MS4A1 (CD20). TMEM176A and TMEM176B transcripts were found in a minority of blood and tonsil B-cell samples. The expression of MS4A and TMEM176 genes in CD5+ blood B-cells and CD27+ (memory) blood B-cells was examined with similar findings: no detection of MS4A4A or MS4A8B, and low or no detection of transcripts from the remaining genes (Figure 2A).

Data were obtained for 14 blood samples obtained from CLL patients (Table 2) and tested similarly for the expression of the MS4A4A and TMEM176 genes (Figure 2B). MS4A1 (CD20)
transcripts were detected at high levels in every sample, as expected (24). MS4A4A expression, which was undetected in normal B-cells, was detected in a single sample. Notably, MS4A6A transcripts were detected at higher levels in 4/14 of CLL samples than in all but one of the 14 normal blood or tonsil B-cell samples, the single exception being one of the blood CD27+ samples. However, the differences in MS4A6A expression between CLL and normal B-cells were not statistically significant (Figure 2C). MS4A8B was not detected in any CLL sample. Expression of MS4A7 and the TMEM176 genes was either low or undetected in CLL samples, as it was in normal B-cells.

Statistical analysis confirmed that the levels of all MS4A and TMEM176 transcripts in CLL were not different than levels found in normal B-cells (Figure 2C).

The data obtained from normal primary B-cells indicated some expression of MS4A6A and MS4A7 in the majority of the samples tested (Figure 2A). In order to assess the potential for the corresponding MS4A proteins to hetero-oligomerize with CD20, we expressed them as GFP fusion proteins in the human BJAB cell line and examined their subcellular localization by fluorescence microscopy. GFP-tagged MS4A4A, MS4A8B, and CD20 were expressed for comparison. Cells were fixed and nuclei stained with DAPI. GFP-MS4A4A and GFP-MS4A8B, like GFP-CD20, appeared to be expressed at the plasma membrane (Figure 3A) and this was confirmed by visualizing the plasma membrane using anti-CD20 (Figure 3C). In contrast, GFP-MS4A6A and GFP-MS4A7 were localized intracellularly in the perinuclear space (Figure 3A).

To characterize the intracellular compartment, we stained early endosomes, cis-Golgi, cis/medial Golgi, and trans-Golgi compartments using antibodies detecting EEA1, golgin 97, golgin 95, and giantin, respectively. Both GFP-MS4A6A and GFP-MS4A7 co-localized with giantin, indicating that they were concentrated in the trans-Golgi complex (Figure 3B); no colocalization with EEA1, golgin 97, or golgin 95 was observed (data not shown).

**Table 2 | Chronic lymphocytic leukemia patients.**

| Age | Gender | Months since diagnosis | WBC count | RAI stage | Prior treatment |
|-----|--------|------------------------|-----------|-----------|----------------|
| 01  | 55 F   | 1                      | 9.5       | 1         | No             |
| 02  | 62 M   | 0                      | 8         | 1         | No             |
| 03  | 71 F   | 0                      | 5         | 0         | No             |
| 04  | 58 F   | 1                      | 13        | 0         | No             |
| 05  | 69 M   | 0                      | 5         | 0         | No             |
| 06  | 78 F   | 0                      | na²       | na²       | No             |
| 07  | 66 M   | 0                      | 12        | 2         | No             |
| 08  | 47 F   | 50                     | 24        | 0         | No             |
| 09  | 83 F   | 0                      | 79        | 0         | No             |
| 10  | 79 M   | 0                      | 12        | 1         | No             |
| 11  | 49 F   | 0                      | 10        | 0         | No             |
| 12  | 54 M   | 0                      | 33        | 0         | No             |
| 13  | 71 M   | 0                      | 20        | 0         | No             |
| 14  | 58 F   | 0                      | na²       | 1         | No             |

Data obtained from Calgary Laboratory Services with ethical approval.

1 Months between diagnosis and time of testing; na, not available.

**DISCUSSION**

This report describes the results of our investigation into the expression of MS4A4A, MS4A6A, MS4A7, MS4A8B, and CD20 in the majority of the samples tested (Figure 2A). In order to assess the potential for the corresponding MS4A proteins to hetero-oligomerize with CD20, we expressed them as GFP fusion proteins in the human BJAB cell line and examined their subcellular localization by fluorescence microscopy. GFP-tagged MS4A4A, MS4A8B, and CD20 were expressed for comparison. Cells were fixed and nuclei stained with DAPI. GFP-MS4A4A and GFP-MS4A8B, like GFP-CD20, appeared to be expressed at the plasma membrane (Figure 3A) and this was confirmed by visualizing the plasma membrane using anti-CD20 (Figure 3C). In contrast, GFP-MS4A6A and GFP-MS4A7 were localized intracellularly in the perinuclear space (Figure 3A).

To characterize the intracellular compartment, we stained early endosomes, cis-Golgi, cis/medial Golgi, and trans-Golgi compartments using antibodies detecting EEA1, golgin 97, golgin 95, and giantin, respectively. Both GFP-MS4A6A and GFP-MS4A7 co-localized with giantin, indicating that they were concentrated in the trans-Golgi complex (Figure 3B); no colocalization with EEA1, golgin 97, or golgin 95 was observed (data not shown).

**FIGURE 3 | Intracellular localization of GFP-tagged MS4A6A and MS4A7.** BJAB cell lines were derived that express GFP-tagged MS4A4A, MS4A6A, MS4A7, MS4A8B, and CD20. (A) Immunofluorescence images are of individual cells representative of > 100 cells observed in at least two independent experiments for each cell line. Cells were fixed and nuclei stained with DAPI. In the case of GFP-MS4A6A and GFP-MS4A7 the cells were counterstained with mouse anti-human CD20 (2H7) and Cy3-conjugated Fab(′)l anti-rabbit IgG to delineate the plasma membrane. (B) Cells expressing GFP-MS4A6A or GFP-MS4A7 were fixed, permeabilized, and stained with rabbit anti-giantin and Cy3-conjugated anti-rabbit IgG to mark the trans-Golgi. Control experiments with non-specific primary antibody confirmed the specificity of the fluorescent signals obtained. Single-labeled control samples were imaged separately to confirm negligible bleed-through of fluorophores to the other channel. Nuclei were stained with DAPI. Results are representative of > 100 cells observed in at least two independent experiments. (C) Cells expressing GFP-MS4A4A or GFP-MS4A8B were stained for CD20 as in (A).
unlikely that very low levels of mRNAs would generate high levels of membrane proteins. We could not examine endogenous MS4A6 and MS4A7 as reliable antibodies were not available, however we found that neither of these proteins localized to the plasma membrane when expressed as GFP fusion proteins in a human B-cell line. This is unlikely to be an artifact of GFP fusion as a similarly constructed GFP-CD20 protein was properly localized to the plasma membrane, as were GFP-MS4A4A and GFP-MS4A8B. Known intracellular localization of MS4A3 (HT1m4) provides a precedent for intracellular localization of some other MS4A proteins (29). It is possible that MS4A6A and/or MS4A7 are mobilized to the plasma membrane under certain conditions, however, our results suggest that neither MS4A6A or MS4A7 is found in sufficiently normal or in the right subcellular compartment to normally form hetero-oligomers with CD20 or to be considered as an alternate therapeutic target for B-cell depletion.

**Table 2**

Presented with RAI stage II CLL at the time of diagnosis when the high level in one CLL sample. This sample was from a patient who was never detected in blood or tonsil B-cells, was expressed at a high level in one CLL sample. This sample was from a patient who presented with RAI stage II CLL at the time of diagnosis when the sample was collected (Table 2), however studies on much larger, defined patient populations would need to be performed to determine the significance, if any, of this observation. MS4A6A was more highly expressed in about 1/3 of CLL samples than in any of the normal B-cell samples, but this difference did not reach statistical significance (Figure 2C). Our study was not designed to test the potential correlation of differential MS4A/TMEM176 gene expression with clinical indicators of prognosis and no conclusions about the significance of these observations can be made at this time. However, testing the protein expression of MS4A4A and MS4A6A in a large cohort of defined CLL samples would be an important direction when antibodies suitable for flow cytometry become available.

In conclusion, we searched for and did not find evidence for MS4A4A, MS4A6A, MS4A7, MS4A8B, or TMEM176 transcripts likely to generate proteins in sufficient quantity to form hetero-oligomers with the abundant CD20 molecules expressed in all normal mature B-cells. MS4A4A and MS4A8B were not detected. MS4A6A and MS4A7 transcripts were detected in most normal B samples but at very low levels and the corresponding proteins are probably not normally expressed at the plasma membrane. TMEM176A and TMEM176B transcripts were only detected in a minority of B-cell samples. Available data for other MS4A genes shows expression restricted to myeloid-lineage cells, testis, lung, and intestinal tissues. Therefore, in the absence of evidence of other MS4A protein co-expressed with CD20 at the plasma membrane, it is most likely that CD20 normally exists in homo-oligomeric form and that alternate therapeutic targets for B-cells are unlikely to be found in the MS4A family. Novel targets are more likely to be found among members of the unrelated but structurally similar tetranspan superfamilly (31). Although broad tissue expression of tetranspan proteins such as CD9 and CD81 limits their potential in this regard, high expression of CD37 in B-cells makes it a promising target for CLL and other B-cell cancers (31–34).

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