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TG2 expressed in mast cells recruited into skin or bone marrow induces the development of pediatric mastocytosis

Young Min Ahn^{1,#}, Gwan Ui Hong^{2,#}, Sang-Hoon Kim³, Ho-Jung Lee⁴, Baek Heysung⁵, Myung Nam Kim⁶, Kui Young Park⁶, Jai Youl Ro^{2,*}

¹Department of Pediatrics, ³Department of Internal Medicine, ⁴Department of Pathology, Eulji Hospital, Eulji University School of Medicine, Seoul, ²Department of Pharmacology and SBRI, Sungkyunkwan University School of Medicine, Suwon, Korea, ⁵Department of Pediatrics, Hallym University Kangdong Sacred Heart Hospital, ⁶Department of dermatology, Chung-Ang University College of Medicine

[#]These authors contributed equally to this work.

Short title: Mastocytosis by mast cell-expressed TG2

***Corresponding author's address:** Jai Youl Ro, Department of Pharmacology, Sungkyunkwan University School of Medicine, Suwon, Korea.

Tel: 82-31-299-6191, Fax: 82-31-299-6209, E-mail: jyro426@med.skku.ac.kr

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Abbreviations: BM, bone marrow; CCL2, chemokine ligand 2; CCR2, chemokine receptor 2; CM, cutaneous mastocytosis; ELISA; enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; H&E, hematoxylin and eosin; IHC, immunohistochemistry; LTC₄, leukotriene C₄; MRI, magnetic resonance imaging; SM, systemic mastocytosis; NF-κB, transcription factor nuclear factor-κB; UP, urticaria pigmentosa; TG2, transglutaminase 2; Treg cells, regulatory T cells; VCAM-1, vascular cell adhesion molecule-1.

Abstract

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Background: Mastocytosis is characterized by a pathological increase of mast cells in organs such as skin and bone marrow. Transglutaminase 2 (TG2) expressed in mast cells contributes to allergic diseases, but its role in mastocytosis has not been investigated. This study aimed to investigate whether TG2 contributes to pediatric mastocytosis.

Methods: Serum, various skin tissues or BM biopsy and aspirates were obtained from pediatric normal control or patients with indolent systemic mastocytoma (SM), mastocytoma and urticaria pigmentosa (UP). Tryptase, individual cytokines, leukotriene C₄ (LTC₄), and TG2 activity in the serum were determined by ELISA, mast cell population by May-Grünwald Giemsa, CD 117 by immunofluorescence, cell surface molecules by Western blot, and co-localization of c-kit and TG2 or IL-10-expressing cells, CD25 and FOXP3 by IHC.

Results: Infiltration of CD25⁺CD117⁺CD2⁻ mast cells into BM and scalp/trunk/ear dermis, expression of FcεRI, tryptase, c-kit, FOXP3, CCL2/CCR2 and VCAM-1, and co-localization of c-kit and TG2 was enhanced in patient's skin tissues or BM, particularly SM, but co-localization of c-kit and IL-10-expressing cells was decreased versus normal tissues. Amounts of LTC₄ and inflammatory cytokines, expression of tryptase or TG2 activity were increased in patient's serum, BM aspirates or ear/scalp skin tissues, respectively, versus normal persons, but IL-10 level was decreased.

Conclusion: The data suggest that mast cells, recruited in the skin and BM by CCL2/CCR, may induce the development of pediatric mastocytosis through reducing IL-10 due to up-regulating TG2 activity via NF-κB. Thus, TG2 may be used in diagnosis of pediatric mastocytosis, particularly SM.

Key words: Cutaneous mastocytosis; recurrent facial nerve palsy; mastocytoma, systemic mastocytosis, tryptase; urticaria pigmentosa; transglutaminase 2 (TG2); c-kit; IL-10.

Name and address of the author to whom request for offprints should be sent Jai Youl Ro, Department of Pharmacology, Sungkyunkwan University School of Medicine, 300 chunchun-dong Jangan-ku, Suwon-440-746, Korea.

Introduction

Mastocytosis refers to a heterogenous group of disorders characterized by an abnormal accumulation of mast cells in skin and internal organs. Mastocytosis is subdivided into cutaneous mastocytosis (CM) and systemic mastocytosis (SM), which is exceedingly rare in

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pediatrics (1, 2). SM affects a variety of organs, such as skin, bone marrow and gastrointestinal tract, and CM is limited to the skin lesions that are subdivided into maculopapular cutaneous mastocytosis (urticaria pigmentosa), diffuse cutaneous mastocytosis, and solitary mastocytoma (2, 3).

Mastocytosis is suspected as rare underlying cause of idiopathic urticaria (4) or bee-sting anaphylaxis (4), but association with angioedema (6) and orbital involvement (7) is rare. There has been no report of facial nerve palsy associated with mastocytosis (8). The most common symptom in childhood CM (90% of patients), which is usually manifested by 2-years-of-age (9), is skin lesions, which turn to reddening, urticarial swelling and pruritus, caused by mediators released from mast cell activation triggered by temperature change, skin friction, ingestion of hot beverage, spicy foods, or certain drugs (10, 11). Flushing, blister formation, nasal congestion, wheezing, abdominal cramping headache, hypotension, and diarrhea have also been reported (9).

Serum baseline tryptase levels are increased in severe mast cell activation in children with mastocytosis (12), and indolent SM without skin lesions represents a unique subgroup of ISM with a particularly low bone marrow mast cell burden (5).

Mutation in *c-kit*, the gene encoding KIT, plays a major role in the pathophysiology of mastocytosis that may lead to the development of greater numbers of mast cells (13). In most patients with adult SM, mutations in the activation loop of KIT (D816V mutation) are detected in mast cells that display an aberrant CD25⁺ immunophenotype (14), but it is detected in approximately one-third of pediatric mastocytosis (13, 15).

TG2 as a cross-linking enzyme is aberrantly activated in tissues and cells. TG2 regulates the NF- κ B, which itself plays a pivotal role in inflammatory processes (16), and contributes to a variety of disease (16, 17), particularly allergic diseases (18, 19). However, the role of TG2 in the pathogenesis of various mastocytosis has not yet been clarified.

Therefore, we examined whether TG2 influences various mastocytosis. We observed that mast cells recruited in dermis may contribute to the pediatric mastocytosis, particularly SM associated with facial nerve palsy, by up-regulating the expression and activity of TG2.

Methods

All protocols were approved by the IRB of Eulji Hospital, Eulji University School of Medicine (Seoul, Korea). The protocol was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.

Human sera and skin tissues

Sera were obtained from normal persons [three (girl)-, six (girl) and four (boy)-years-old, $n = 3$] and a three (boy)-, five (girl)-, four (boy, $n = 2$)- and ten (girl)-years-old with mastocytosis ($n = 5$) (550 x g, 30 min). Trunk (one SM, two mastocytoma and two UP; three groups used), and scalp and ear skin tissues, or BM aspirates and trephine biopsy (a 10 years-old girl) were obtained from patients with mastocytosis or three pediatric normal persons, who provided sera.

Determination of inflammatory cells, mast cells or surface molecules in skin or BM

The deparaffinized and blocked trunk (three groups), scalp and ear skin tissue sections or BM biopsy (3 μm) and aspirates in SM were stained by H&E for inflammatory cells, and by wright-Giemsa and May-Grünwald Giemsa for mast cells. Numbers of mast cells located within the dermis or epidermis were quantified at 10 sites of 200 $\mu\text{m} \times 200 \mu\text{m}$ areas (10 sites/each slide $\times$ 3 normal tissues or 1 skin tissue with mastocytosis = 30 sites and 10 sites, respectively) under a microscopy (see more details in Data S1) (20).

Immunohistochemistry (IHC) and immunofluorescence

The deparaffinized and blocked trunk (three groups), scalp and ear skin tissue or BM biopsy sections (3 μ m) and smeared aspirates in SM for IHC, and scalp skin tissue sections (3 μ m) in SM for immunofluorescence were performed as described previously (see more details in Data S1) (20).

Immunoblotting

Immunoblotting was performed for protein extracts from homogenized scalp or trunk skin tissues of SM as described previously (see more details in Data S1) (20).

EMSA

Nuclear extracts were prepared from scalp skin tissues of SM (50 mg/500 μ l), and performed using EMSA (see more details in Data S1) (20).

Measurement of tryptase, leukotriene (LT) C₄, cytokines or TG2

Amounts of tryptase, LTC₄ and individual cytokines or TG2 activity in sera of pediatric normal control, SM, mastocytoma and UP were determined by human ELISA kit and TG-Covtest kit, respectively (see more details in Data S1) (20).

Statistical analysis

Experimental data were shown as means $\pm$ SEM. Significance analysis between controls and experimental groups were carried out using the Student *t*-test for unpaired data. Multiple group comparisons were performed using one-way ANOVA followed by Scheffe's *post hoc* test using SPSS (SPSS Inc., Chicago). *P* values < 0.05 were regarded as significant. The densitometry analysis of Western blots and EMSA was performed with Quantity One

(version 4.6.3; Bio-Rad, Hercules, CA) and are indicated as means $\pm$ SEM (n = 4) obtained from the ratio of each band density versus those in the control and loading control of four independent experiments.

Results

Clinical finding for histological changes of patient

A 10 years-old girl presented with recurrent peripheral facial palsy and erythematous nodules on periorbital area (see more details in Data S2) (Fig. 1A left and middle box).

Her physical examination (the 10 years-old girl, SM) revealed two erythematous plaques on the scalp and brownish macules over the extremities and trunk (Fig. S1Aa and Ba, respectively). Her blood counts and biochemical profiles except to increment of serum tryptase (81.7 ng/ml versus normal values, < 11 ng/ml) were within normal, and oral challenge with ibuprofen was positive (see more details in Data S2).

Her histological examination of a biopsy taken from the scalp and trunk lesions revealed dense infiltration of inflammatory cells (Fig. S1Ab and Bb, respectively), and they were almost mast cells which expressed a CD117 (Fig. S1Ac and Bc, respectively).

As she showed high tryptase, which was associated with SM (9), Bone marrow (BM) examination (BM aspiration and biopsy) were required for further evaluation. The BM biopsy showed multifocal clusters of CD25⁺CD117⁺CD2⁻ mast cells (Fig. 1B and C), which showed increased tryptase in BM aspirates (Fig. 1C right) and scalp skin (Fig. 1E left). Thus, we finally diagnosed indolent SM (10 years-old girl) with recurrent nerve palsy, although we failed to get the results for genetic KIT mutation from BM aspirates. Further details of her

clinical history, gene examination, the data explanation for BM biopsy/aspirates, and recurrent facial nerve palsy, which has been no report for association with mastocytosis (8, 21, 22) (Fig. 1A right box, see yellow circle; Fig. S1C), were described in Data S2.

Two boys of patients showed as a yellowish nodule, and one girl and one boy of patients showed generalized eruption of red-brown macules or papules on trunk in the physical examination. Their blood counts and biochemical profiles as well as total IgE levels (55 IU/ml for two boys having a yellowish nodule and 87 IU/ml for both girl and boy versus normal, 1-183 IU/ml) were within normal, but only serum tryptase was increased (42.2 ng/ml for two boys; 48.3 ng/ml for both girl and boy versus < 11 ng/ml). However, it increased less than that in 1 10 years-old girl. The c-kit point mutation is not found in four patients with mastocytosis. Thus, we also diagnosed as mastocytoma for 3 and 4 years-old boys and UP for 4 (boy)- and 5 years (girl)-old.

Population, tryptase, receptor, markers or TG2 of mast cells in skin tissues

As BM biopsy is difficult to get from a child, we used biopsy of scalp skin of patient with SM or trunk skin of patients with mastocytosis (n = 2) and UP (n = 2) in all the next experiments. Numbers of mast cells (from 6 ± 3.2 cells to 38 ± 9.8 cells) were remarkably increased in a scalp dermis of patient with SM (Fig. 1D). Mast cells were not significantly increased in epidermis (from 8 ± 4.2 to 13 ± 5.4 cells) versus those in normal tissues. Population of mast cells was also increased in mastocytoma (from 4 ± 3.4 cells to 21 ± 5.5 cells) or UP (to 26 ± 7.3 cells) versus normal tissues, but it showed much less than that in SM (Fig. 1E).

In order to re-confirm whether the cells infiltrated in dermis of SM scalp were mast cells or related to mast cells, we observed that expression of FcεRI receptor and markers (c-kit and tryptase) in mast cells, transcription factor (FOXP3) of Treg cells, TG2, and migration (CCL2/CCR2) and adhesion (VCAM-1) molecules were increased in scalp skin tissues of patient with SM versus normal (Fig. 2A).

TG2, aberrantly activated in tissues and cells, regulates the NF-κB (19) and contributes to allergic diseases (20). However, the role of TG2 in the pathogenesis of pediatric mastocytosis has not yet clarified.

NF-κB activity (Fig. 2B), which plays a pivotal role in the inflammatory processes and regulates TG2 (19), or amount of LTC₄ (Fig. 2C right column) and various inflammatory cytokines (Fig. 2D), which were related to symptoms of mastocytosis, were remarkably enhanced in patient skin tissues and serum, respectively, of patients with SM by approximately 5-fold, compared to normal. Amounts of LTC₄ or various cytokines in SM were increased more than those in mastocytoma or UP, but there were no statistical significance among three groups. Only IL-10 among various cytokines was decreased in serum of patients with mastocytosis of three groups.

TG2 activity or co-localization of mast cells and TG2 in skin tissues

TG2 as a catalytic enzyme is associated with the allergic asthma (19), and mast cells induce the expression/activity of TG2 (20). Appropriately, TG2 activity, together with expression of TG2 in BM aspirates (Fig. 1C right column) and scalp skin (Fig. 2A), was remarkably enhanced in serum of a patient with SM versus normal (Fig. 2C left column), and it showed the significant difference, compared to that in mastocytoma or UP.

Co-localization (merge, yellow) of mast cell surface marker c-kit (green color) and TG2 (red) was shown in dermis of patients with SM (28 ± 4.2 cells), mastocytoma (10 ± 3.6 cells) or UP (12 ± 2.8 cells) versus normal tissues (1 ± 0.3 cells) (Fig. 3A). However, co-localization in SM was remarkably increased in skin tissues, compared to mastocytoma or UP. Thus, TG2 expressed in mast cells may contribute to the development of various mastocytosis. However, it needs to further investigate a role of TG2 in BM of patients with mastocytosis in three groups.

As IL-10 level, which are associated with Treg cell suppressive function, was reduced in the serum of patient with mastocytosis, we examined the population of Treg cells (CD25 and FOXP3) in skin tissues of the patient with SM. Co-localization (merge) of surface marker CD25 and transcription factor FOXP3 of Treg cells was increased in dermis of patient with SM (Fig. S1D). However, co-localization of c-kit and IL-10-expressing cells was decreased more in patient scalp dermis rather than normal skin (Fig. 3B).

DISCUSSION

Our data demonstrate that mast cells, infiltrated and activated into BM and skin tissues through CCL2/CCR2 and VCAM-1 up-regulated by various foods or medications, may express/activate TG2, which reduces IL-10 production, in pediatric mastocytosis, particularly SM with recurrent facial nerve palsy, followed by mediator release (LTC_4 and cytokines) associated with the development of pediatric mastocytosis via NF- κ B activity.

Even though the etiology is unknown, many forms of mastocytosis are associated with point mutations in the genes coding for growth factor receptor c-kit, but c-kit mutation is rarely reported in children with UP (23). This mutation was not detected in this study. Sometimes,

UP may progress to systemic mastocytosis, which is especially associated with a high tryptase level (9, 24). Serum tryptase levels in SM were persistently elevated, but there has been no further reported evidence of SM. Further close observation is needed. However, among five pediatric patients, one of them was diagnosed as an indolent SM with recurrent peripheral nerve palsy, two of them as mastocytoma, and two of them as UP. As demonstrated by the data for clinical history, biochemical profiles including high tryptase levels in SM (9), and by the data explanation for BM biopsy/aspirates and recurrent facial nerve palsy, we focused on SM. Thus, both pictures for mastocytoma and UP were not shown.

Accumulation of mast cells in the skin may present as maculopapular CM, diffuse CM, and mastocytoma (21). Urticaria with angioedema is uncommon, and recurrent prominent urticaria should prompt consideration of other allergic disorders (6). However, mastocytosis is suspected as a rare underlying cause of idiopathic urticaria or bee-sting anaphylaxis (4, 5, 25).

Clinical symptoms of mastocytosis arising from mediators released in mast cell degranulation are vasodilatation, hypotension, flushing, pruritus, and gastrointestinal complaints, which are exacerbated by temperature change, skin friction, ingestion of hot beverage or spicy foods, alcohol, or certain drugs (9, 26). In this study for SM, symptoms including itching, flushing, swellings on vulva and periorbital area may have been precipitated by mediators released from mast cell activation by various foods and medications. Mastocytoma and UP may also be caused by mediators released from mast cell activation.

Mast cells migrate into inflammatory sites by up-regulating the migration molecules (CCL2/CCR2) and adhesion molecules (VCAM-1 and IL-8) expressed by various stimuli (19, 27). TG2 is expressed and activated in mast cells related to allergic asthma (20), and the expressed TG2 induces the expression of inflammatory cytokines through NF- κ B activity. TG2 also induces mediator release by activities of MAP kinases and PLA₂ (19, 20). In this study, the data can infer that mast cells may migrate into dermis through up-regulation of CCL2/CCR2 and VCAM-1, and the migrated mast cells may induce the expression/activation of TG2. TG2 may also enhance the expression of inflammatory cytokines via NF- κ B activity as well as other mediators, such as histamine and LTC₄. Thus, TG2 expressed in mast cells may contribute to the development of pediatric mastocytosis, particularly SM, as demonstrated by the data for co-localization of mast cells and TG2 in skin tissues of the patient with mastocytosis.

Treg cell suppressive function is programmed by transcription factor FOXP3 (27, 28), and is associated with IL-10 and/or TGF- β (27). However, in this data, FOXP3⁺-Treg cells and TGF- β were increased in the patient's skin tissues, but IL-10 was more decreased than in normal skin tissues. These results have several implications. First, the infiltration of mast cells into patient's skin tissues may be correlated with the increase of the number of Treg cells, as shown by the correlation of increased numbers of gastric-infiltrated mast cells with number of Treg cells in gastric cancer patients (30). We speculate that one of ways in which mast cells affect the Treg cells is their secretion of TGF- β , which may induce FOXP3⁺-Treg cells. Second, the finding that FOXP3⁺-Treg cells are increased in mastocytosis may be exploited in curbing induction of this disease.

The reason that population of IL-10-expressing cells is decreased in mastocytosis may reflect the fact that mast cells, which are a counterpart of Treg cells, are much more abundant than Treg cells in patient's skin tissues. However, further studies should be performed to explore the mechanism of correlation of mast cells, Treg cells, and IL-10-expressing cells in mastocytosis.

In conclusion, our study reports a case of SM presenting recurrent angioedema and facial nerve palsy, which is considered in the differential diagnosis of recurrent angioedema. Our data also suggest that TG2, which is increased in SM much more than those in mastocytoma and UP, and expressed/activated in mast cells, may play an important role as a promising marker to diagnose pediatric mastocytosis, particularly SM with urticaria pigmentosa, presenting recurrent periorbital angioedema and facial nerve palsy in Korea.

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Conflict of interest

The authors state no conflict of interest.

Author contributions

Drs Ahn and Ro have participated sufficiently in the work of manuscript. Ahn, Baek, Kim³, Lee and Park diagnosed pediatric mastocytosis and analyzed data; Hong and Kim⁶ performed

molecular research and briefly wrote the paper; Ahn and Ro designed research, analyzed data and wrote the paper.

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Legends for figures

Figure 1. Clinical symptoms and histological changes in BM biopsy/aspirates, population and tryptase of mast cells in the skin tissues of pediatric patients. (A) Picture for facial nerve palsy (left box), recurrent periorbital angioedema and chemosis (middle), and brain image in patients with SM by MRI (right). (B) Expression of CD2 (BM aspirates), CD25 or CD117 in BM biopsy of patients with SM using IHC. (C) Population of mast cells in BM using H&E (biopsy) and wright-Giemsa (aspirates, blue color), and expression of tryptase and TG2 in BM aspirates using IHC (brown). (D) Mast cell population (blue color) in SM scalp skin tissues using May-Grünward Giemsa. (E) Expression of tryptase in SM scalp skin tissues (left, brown color) by IHC. (F) Population of mast cells in mastocytoma and UP using May-Grünward-Giemsa. Values (numbers of cells) in Fig. 1D and 1F indicated in box. Small box in right upper corner indicates single mast cell enlarged with magnification x1000. Arrows indicate mast cells. Magnification in Fig. 1B was x400 and in short- or long-scale bar in Fig. 1C and D was x200 and x1000, respectively. Magnification in Fig. 1E and 1F was x200, and all scale bars were 100 μ m.

Figure 2. Expression of surface and migration molecules, NF- κ B activity in scalp or trunk tissues, or TG2 activity, LTC₄ or cytokines in serum of patients with mastocytosis. (A) Expression of surface/adhesion molecules in SM scalp skin tissues by Western blot. #, Numbers below bands show the mean obtained from three normal (n = 3) or from three individual experiments of one patient (n = 1). (B) NF- κ B activity in SM scalp skin tissues by

EMSA. (C) TG2 activity and amount of LTC₄ in sera by ELISA (n = 4). (D) Amounts of various cytokines by ELISA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the normal. +, $P < 0.05$; ++, $P < 0.01$ versus SM. Masto, mastocytoma.

Figure 3. Co-localization of mast cells and TG2 or IL-10-expressing cells in scalp or trunk tissues of patients with SM, mastocytoma or UP. (A) Co-localization (yellow) of mast cell surface marker c-kit (green) and TG2 (red) by immunofluorescence. (B) Co-localization of mast cells and IL-10-producing cells (red). The values (cell counting) for co-localization in box were quantified in a 200 x 200 μm areas (10 sites/each slide x 3 individual for normal or 2 patient = 30 areas and 20 areas, respectively). Magnification was x400 and all scale bars were 100 μm .

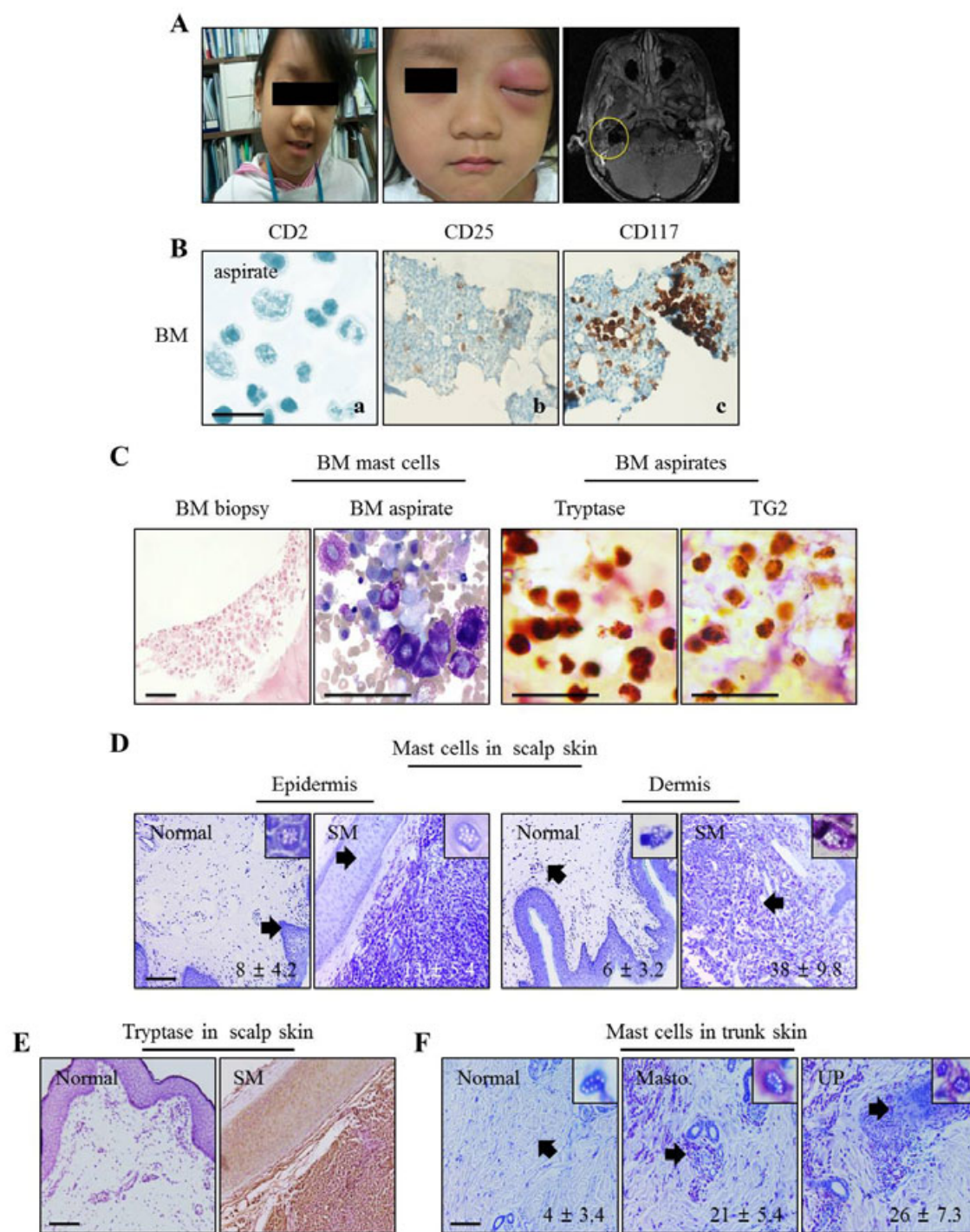
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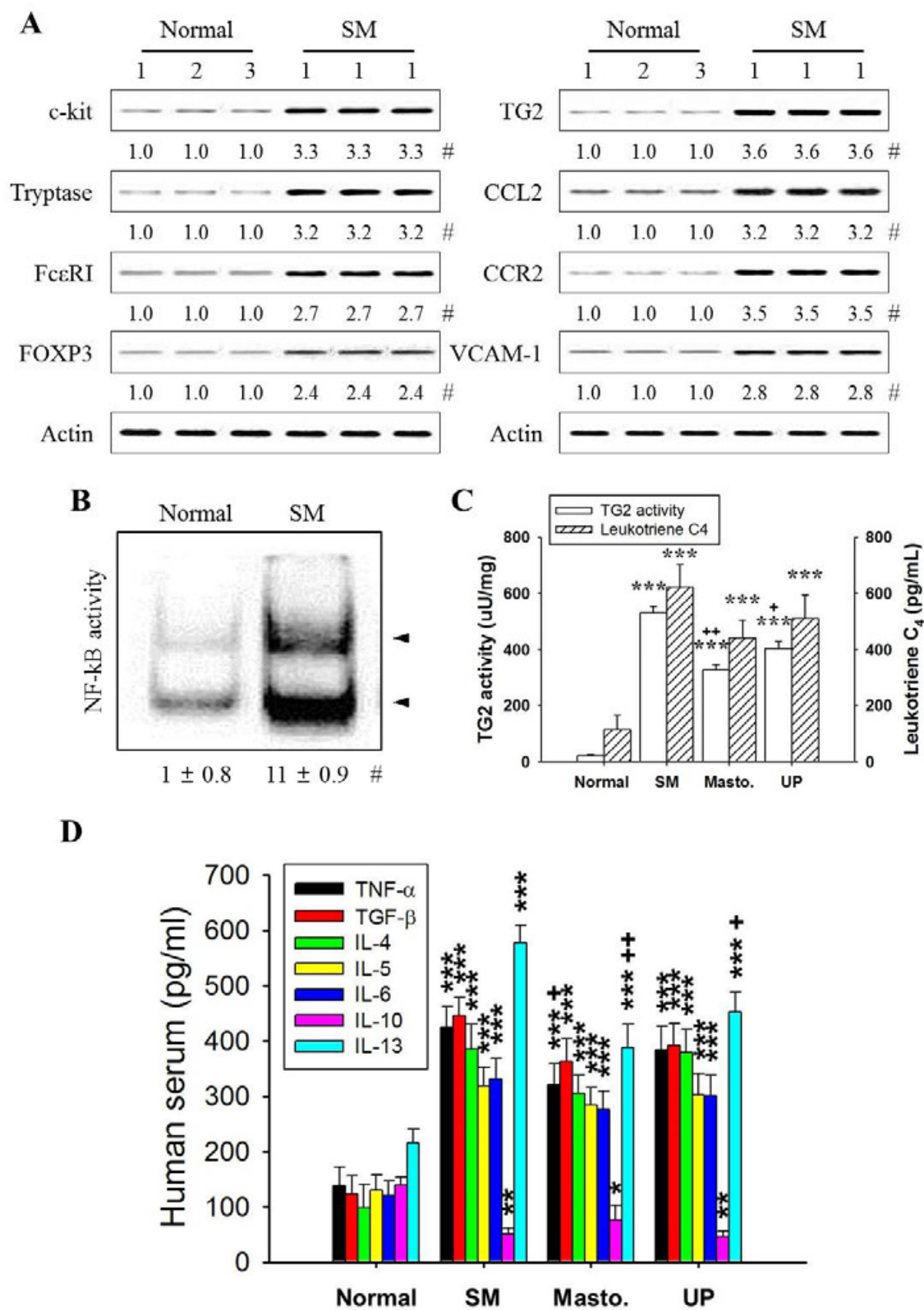
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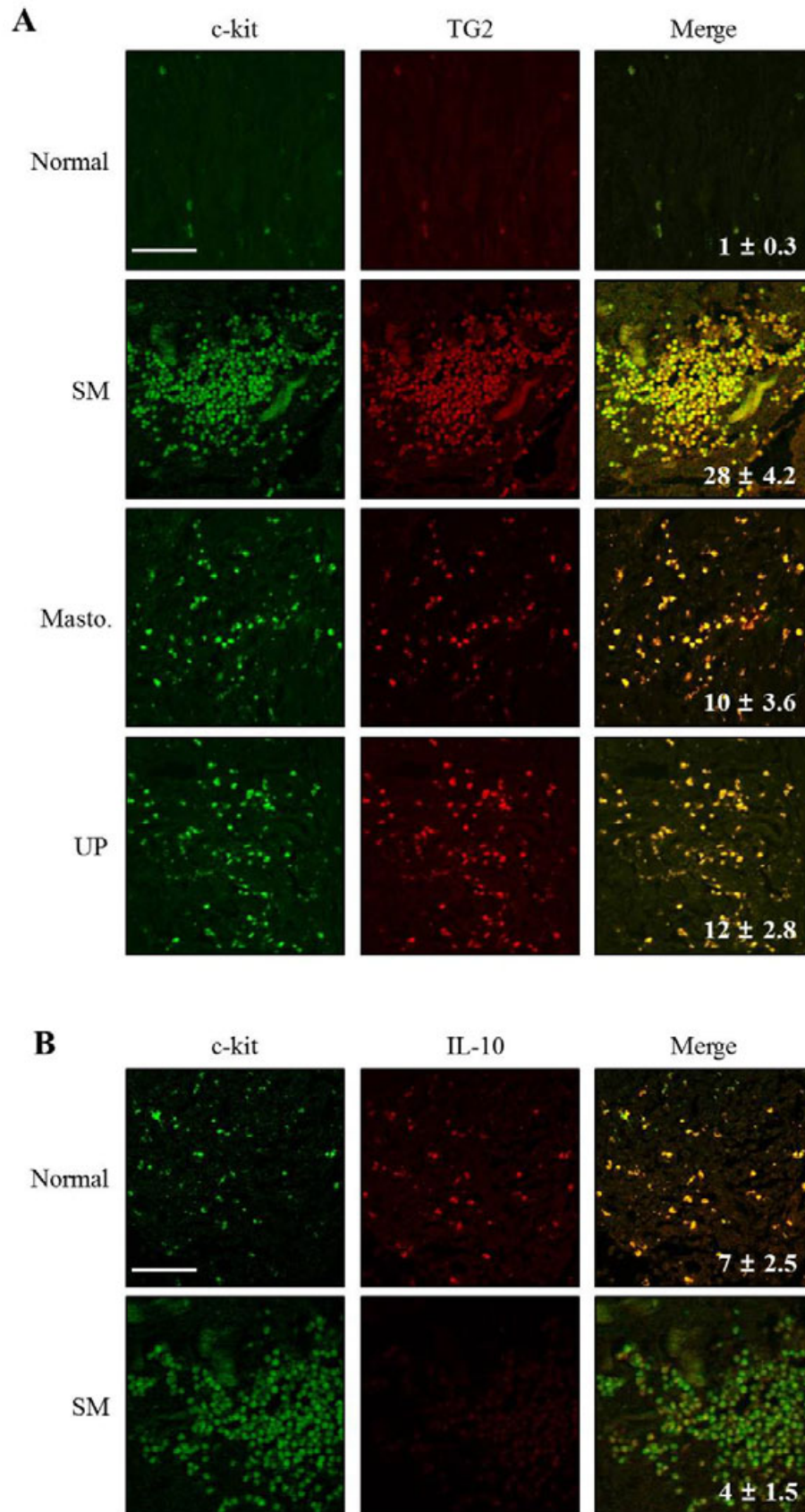
Data S1. Methods

Data S2. Results

Figure S1. Clinical symptoms and histological changes, inflammatory cells and expression of surface molecules of mast cells or co-localization of CD25 and FOXP3 in the various skin tissues.







Online Supplementary Data

TG2 expressed in mast cells recruited into skin or bone marrow induces the development of pediatric mastocytosis

Young Min Ahn^{1,#}, Gwan Ui Hong^{2,#}, Sang-Hoon Kim³, Ho-Jung Lee⁴, Baek Heysung⁵,
Myung Nam Kim⁶, Kui Young Park⁶, Jai Youl Ro^{2,*}

¹Department of Pediatrics, ³Department of Internal Medicine, ⁴Department of Pathology, Eulji Hospital, Eulji University School of Medicine, Seoul, ²Department of Pharmacology and SBRI, Sungkyunkwan University School of Medicine, Suwon, Korea, ⁵Department of Pediatrics, Hallym University Kangdong Sacred Heart Hospital, ⁶Department of dermatology, Chung-Ang University College of Medicine

[#]These authors contributed equally to this work.

Short title: Mastocytosis by mast cell-expressed TG2

***Corresponding author's address:** Jai Youl Ro, Department of Pharmacology and SBRI, Sungkyunkwan University School of Medicine, Suwon, Korea.

Tel: 82-31-299-6191, Fax: 82-31-299-6209, E-mail: jyro426@med.skku.ac.kr

Methods

Determination of inflammatory cells or mast cells in skin tissues or bone marrow (BM)

Trunk (SM, mastocytoma and UP), scalp, and ear skin tissues, or BM biopsy and aspirates obtained by biopsy or aspiration in SM (a 10 years old girl), respectively, were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 μ m. The deparaffinized and blocked skin tissue and BM biopsy sections or BM aspirates stained by H&E for inflammatory cells including mast cells (20), and by wright-Giemsa and May-Grünwald Giemsa (Merck, Darmstadt, Germany) for mast cells. After that, numbers of mast cells located within the dermis or epidermis were quantified at 10 sites of 200 μ m $\times$ 200 μ m areas (10 sites/each slide $\times$ 3 normal tissues or 1 skin tissue with mastocytosis = 30 sites and 10 sites, respectively) under a microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany) (20).

Immunohistochemistry (IHC) and Immunofluorescence

For IHC, the deparaffinized and blocked trunk (three groups), scalp and ear skin tissue or BM biopsy sections (3 μ m) and BM smeared aspiration in SM coated with a primary Abs against anti-human CD2, CD25 or CD117/kit, tryptase or TG2 (1:50, Abcam, Cambridge, MA) for overnight, and followed by staining with Dako EnVision Plus-peroxidase detection kit (Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin and finally mounted using aqueous mounting medium (Thermo Shandon, Pittsburgh, PA), and then quantified under a light microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany) (20).

For immunofluorescence, scalp skin tissue sections (3 μ m) in SM were incubated with the Abs against c-kit and transglutaminase 2 (TG2), CD25 and FOXP3 or c-kit and IL-10 in blocking solution for overnight (all Abs' dilution, 1:25). The slides were treated with the corresponding FITC- or TEXAS-conjugated anti-IgG (1:100, Zymed Laboratory Inc., San

Francisco, CA) for 1 h in room temperature. Washing steps between each stage were performed for 1-2 min. After washing, the slides were mounted using aqueous mounting medium (Thermo Shandon, Pittsburgh, PA), and quantified by Confocal microscope (Carl Zeiss, Oberkochen, Germany) (20)

Immunoblotting analysis

Skin tissues of SM were suspended in a low-salt lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 µg/ml aprotinin, 2.0 µg/ml leupeptin] and allowed to swell on ice for 30 min. The tissues were then homogenized using a Polytron (Kinematica, Littau, Switzerland). Supernatants (20 µg) obtained after centrifugation (550 x g) were subjected to 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were washed with PBS containing 0.1% Tween 20 (PBST), and then blocked for 1 h in 5% skim milk in PBST. After the membranes were washed with PBST, they were incubated for 60 min at room temperature with primary Abs against c-kit, FcεRI (Santa Cruz Biotechnology, Santa Cruz, CA), tryptase (Abcam, Cambridge, MA), FOXP3, TG2, CCL2, CCR2, VCAM-1 (Cell Signaling Technology, Beverly, MA). Membranes were washed with PBST and treated with HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG (diluted to 1:5,000 ~ 1:10,000) (Bethyl Laboratories, Montgomery, TX) in PBST for 60 min. After washing, the protein bands were visualized by ECL using a chemiluminometer (Amersham Biosciences, Buckinghamshire, UK) (20).

EMSA

Nuclear extracts were prepared from scalp skin tissues of SM (50 mg/500 µl), and then EMSA were performed as described previously (20). Ten µl of a mixture of NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5') oligonucleotide (1.75 pmol/µl) (Promega Corporation), T4 polynucleotide kinase 10 X buffer, [α -³²P]ATP (10 µCi; 3,000 Ci/mmol), nuclear-free water, and T4 polynucleotide kinase (5-10 U/µl) were incubated at 37 °C for 30 min. After adding 89 µl Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], unincorporated nucleotides were removed from the DNA probe by chromatography through a G-25 spin column (Amersham Biosciences, Buckinghamshire, UK). The nuclear extract (4 µl) and gel shift binding 5X buffer (6 µl) [20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50mM Tris-HCl (pH 7.5), 0.25 mg/ml poly (dI-dC)] were incubated at room temperature for 10 min, and then 20-30 fmol of ³²P-labeled NF-κB oligonucleotide (9 µl) was added, and incubated at room temperature for 20 min. After stopping the reaction, 1 µl of 10 X gel loading buffer was added to each reaction. Reaction mixtures (20 µl) were electrophoresed on 6% polyacrylamide gels, and the gels were analyzed using FLA-2000 (FUJIFILM, Tokyo, Japan).

Measurement of tryptase, leukotriene C₄ (LTC₄) or cytokines in sera

Tryptase, or individual cytokines in sera of pediatric normal control, SM, mastocytoma and UP were determined by human ELISA kit (Antibodies-Online, Atlanta, GA and Life technologies, Gaithersburg, MD, respectively). Briefly, micro-plates were coated with 200 µl purified anti-human tryptase or individual cytokines (TNF-α, TGF-β, IL-4, 5, 6, 8, 10, 13) for overnight, and treated with human sera (100 µl) or standards. After incubation for 2 h, each

well was treated with 100 μ l biotin conjugated antibody. After that, the concentration of tryptase or cytokines was measured by adding a 50 μ l chromogenic substrate. The resultant color intensity was read at 450 nm using an ELISA plate reader (20). The lowest detection limit for tryptase was better than 0.39 ng/ml, for IL-8 and TNF- α was better than 15.6 pg/ml, for TGF- β and IL-13 was better than 31.2 pg/ml, and for IL-4, IL-5, IL-6 and IL-10 was better than 7.8 pg/ml.

The LTC₄ contents in sera of pediatric normal control, SM, mastocytoma and UP were determined using ELISA (Santa Cruz Biotechnology, Inc., Santa Cruz). The concentrations of LTC₄ were then calculated using standard curves generated with specific LTC₄ standards and analyzed using analysis tools on the Cayman Chemical website (<http://www.caymanchem.com/app/template/analysis%2CEIA.vm/a/z>) (20). The results are expressed in pg/ml (1 x 10⁶ cells). The lowest detection limit for TLC₄ was better than 50 pg/ml.

TG2 activity assay

TG2 activity in the sera of pediatric normal control, SM, mastocytoma and UP was determined using the TG-Covtest kit (Covalab, Villeurbanne, France) (20). Briefly, microplates were coated with purified 200 μ l anti-TG2 for overnight, and added with human sera (100 μ l) or TG2 standards, and then incubated for 2 h. After incubation, each well was treated with 100 μ l Streptavidin-horseradish peroxidase (Sav-HRP) and 50 μ l HRP substrate, and then read at 450 nm using an ELISA plate reader. The TG2 activity was calculated using standard curves generated with specific TG2 standard, and expressed in μ U/mg protein. All

117 assays were done in triplicate. The lowest detection limit for TG2 activity was better than
118 0.59 μ U/mg protein.

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Results

Clinical finding for histological changes of patients

A 10 years-old girl has been presented with recurrent unilateral periorbital swelling since four years old. She was admitted under the misdiagnosis of periorbital cellulitis two times, and then idiopathic angioedema was diagnosed. She initially complained of itching and swelling on vulvar area, and then developed unilateral or bilateral erythematous purpuric swelling of periorbital area and chemosis with varying intensities. Sometimes, flushing, abdominal pain and chest tightness were complained. Her symptoms were precipitated by stimuli including various foods (fishballs and candies) and medications (ibuprofen, cimetidine, calcium and vitamin D complex). First episode of right facial hemiparesis were noted with preceding right parotid area swelling, and four more episode occurred on both sides with variable severity. Her symptoms were improved with oral antihistamines and oral steroid during the episodes, but maintenance of antihistamines or leukotriene receptor antagonist was not effective in preventing or relieving the symptoms, and all her symptoms have recurred despite cautious dietary restrictions.

Physical examination and skin test

Rubbing the skin elicited a Darier's sign. There was no evidence of organomegaly or lymphadenopathy on abdominal ultrasonography. Her (the 10 years-old girl, which is inferred to SM) blood cell counts, the biochemical profiles, complement C3/C4, and serum IgE level (< 2.0 IU/ml; normal values, 1-183 IU/ml) were within normal limits. However, serum tryptase levels were remarkably elevated at 4, 8, 9, and 10 years old (52 ng/ml, 73 ng/ml, 30 ng/ml and 81.7 ng/ml, respectively) versus that in normal values (< 11 ng/ml). 25-OH Vit D was 5.2 ng/ml (normal 20-100 ng/ml).

Skin test with common inhalant and food allergens was all negative, and ibuprofen challenge tests were positive (erythematous swelling on forehead developed 10 min after 86 mg cumulative ingestion). Bone densitometry showed low normal range (data not shown). In histological examination of a biopsy taken from the scalp and trunk lesions, epidermis showed increased basal melanin pigmentation. In scalp skin tissues, genetic examination of KIT mutation at codon 816 was negative (no mutation in exons 9, 11, 13, 17 of c-Kit) by sequencing (data not shown). Even though etiology is unknown, many forms of mastocytosis are associated with point mutations in the genes coding for c-kit, but D816V mutation is not frequently reported in pediatric CM (15), explaining its regression after puberty. Sometimes, it may progress to SM (1, 7, 25).

Biopsy and aspirates of bone marrow of a patient with mastocytosis

BM biopsy (the 10 years-old girl) showed multifocal clusters of CD25⁺CD117⁺CD2⁻ mast cells, which is large, round to oval nuclei with clumped chromatin, and a low nuclear/cytoplasmic ratio with abundant cytoplasm (Fig. 1C), consisting about 10-20% of all nucleated hematopoietic elements and 70% cellularity, which was hypocellular for her age. BM aspirates also showed an increased number of large, round, hyper-granulated mast cells without atypia. Based on clinical and histopathological findings and the WHO criteria, we finally diagnosed indolent SM with urticaria pigmentosa, although we failed to get the results for genetic KIT mutation from BM aspirates. However, it needs to further evaluate the genetic KIT mutation for precise diagnosis.

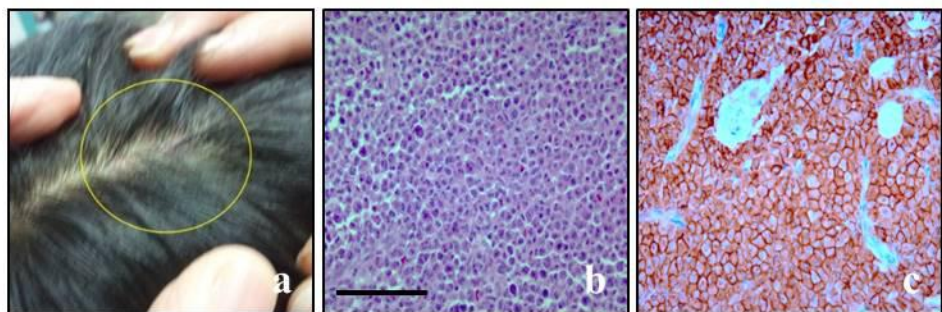
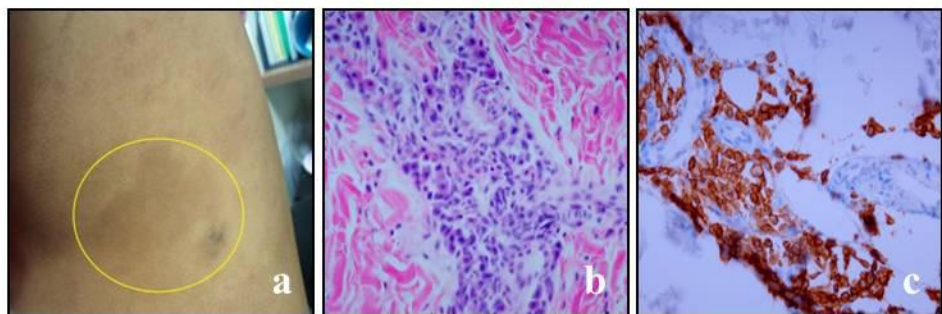
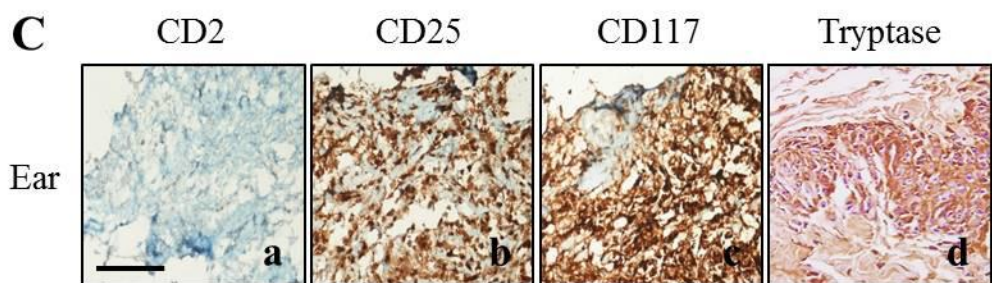
Last facial palsy

Last facial palsy developed at 10 years old, which has been no report for its association with

mastocytosis (8, 21), was appeared at right side of face and prolonged for one month. Gadolinium-enhanced MRI revealed heterogenous signal of both parotid gland which especially showed more severity in right side (Fig 1A right box, see yellow circle) and no neurogenic tumor along 7th and 8th cranial nerve. Temporal bone CT image also suggests the possibility of facial nerve involvement in parotid area. Blink reflex was absent, consistent with defect in efferent pathway and facial nerve conduction study, suggesting the bilateral facial neuropathy which was worse on the right. Right ear canal was narrow due to swelling, and dense infiltration of CD25⁺CD117⁺CD2⁻ mast cells (Fig. S1C) and tryptase was found on ear skin biopsy. However, this case can be excluded Melkersson-Rosenthal syndrome due to lack of development of labial hypertrophy and absence of granuloma, which is characterized by recurrent attacks of Bell's palsy, painless edema of the face, cheilitis granulomatosa and fissured tongue, and diagnosed by histopathologic identification of noncaseating granulomas, caused by unknown delayed hypersensitivity syndrome (22). We need further biopsy from parotid and orbital area to confirm the mast cell infiltration.

183 **Figure Legends**

184 **Figure S1.** Clinical symptoms and histological changes, inflammatory cells and expression of
185 surface molecules of mast cells or co-localization of CD25 and FOXP3 in the various skin
186 tissues. (A) and (B) Scalp and trunk skin tissues, respectively. (Aa) and (Ba) Picture for
187 erythematous nodule suggesting mastocytoma. (Ab) and (Bb) Inflammatory cells infiltrated
188 in skin tissues using H&E. (Ac) and (Bc) c-kit (CD117) in skin tissues of patient with SM by
189 IHC. (C) Expression of CD2, CD25, CD117 and tryptase in ear skin tissues of patient with
190 SM by IHC (brown color). (D) Co-localization (yellow) of Treg cell surface marker (CD25,
191 green) and Treg cell transcription factor FOXP3 (red) in scalp skin tissues of SM by
192 immunofluorescence. The values (cells) for co-localization in box were quantified in a 200 x
193 200 μm areas (10 sites/each slide x 3 individual for normal or 1 patient = 30 areas and 15
194 areas, respectively. Magnification was x400. All scale bars indicated 100 μm .

A**B****C****D**