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Inflammatory expression profile in peripheral blood mononuclear cells from patients with Nasu-Hakola Disease

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ABSTRACT

Homozygous mutations in Triggering Receptor Expressed on Myeloid cells 2 gene (*TREM2*) are one of the major causes of Nasu Hakola Disease (NHD). We analysed Peripheral Blood Mononuclear Cells (PBMC) profile of 164 inflammatory factors in patients with NHD carrying the *TREM2* Q33X mutation as compared with heterozygous and wild type individuals.

Several molecules related to bone formation and angiogenesis were altered in NHD compared to non-carriers: Bone Morphogenetic Protein (BMP)-1 mRNA levels were significantly increased in PBMC (2.32 fold-increase; $P = 0.01$), as were Transforming Growth Factor Beta (TGFB)3 levels (1.51 fold-increase; $P = 0.02$). Conversely, CXCL5 and Pro Platelet Basic Protein (PPBP) were strongly downregulated (-28.26, -9.85 fold-decrease over non-carriers, respectively, $P = 0.01$), as well as Platelet Factor 4 Variant 1 (PF4V1; -41.44, $P = 0.03$).

Among other inflammatory factors evaluated, Interleukin (IL)-15 and Tumor Necrosis Factor Superfamily Member (TNFSF)4 mRNA levels were decreased in NHD as compared with non-carriers (-2.25 and -3.87 fold-decrease, $P = 0.01$ and 0.001, respectively).

In heterozygous individuals, no significant differences were observed, apart from IL-15 mRNA levels, that were decreased at the same extent as NHD (-2.05 fold-decrease over non-carriers, $P = 0.002$).

We identified a signature in PBMC from patients with NHD consisting of strongly decreased mRNA levels of CXCL5, PPBP, PF4V1, mildly decreased IL-15 and TNFSF4 and mildly increased BMP-1 and TGFB3.

1. Introduction

Nasu-Hakola Disease (NHD), also known as Polycystic Lipomembranous Osteodysplasia with Sclerosing Leukoencephalopathy (PLOSL), is a recessively inherited rare disorder characterized by a combination of pre-senile frontal dementia and systemic bone cysts formation, leading to pathological fractures of the wrists and ankles after microtrauma [1–3]. NHD culminates in a profound dementia and death occurs by the age of 50. It is caused by mutations in Triggering Receptor Expressed on Myeloid cells 2 gene (*TREM2*) or TYRO protein tyrosine kinase binding protein (*TYROBP*), also known as DNAX-activating protein of 12 kDa (*DAP12*). These genes encode different domains of the same receptor signaling protein, involved in the activation of the immune response (namely TREM2/TYROBP signaling cascade). TREM2/TYROBP signaling is essential for the development of osteoclasts and dendritic cells [4] as well as for microglia activation, phagocytosis and survival [5].

TREM2 possesses an immunoglobulin superfamily domain [6] and is expressed in myeloid cells including microglia, monocyte-derived dendritic cells and macrophages [7]. It plays important roles in innate and adaptive immunity [8]. TREM2 promotes survival, proliferation and remodeling of the actin cytoskeleton, which regulates adhesion and migration [9]. TREM2 is involved in key signaling events related to the immune response and the phagocytic activity of microglia [10].

In mice models, TREM2 was found to be essential for microglia-mediated synaptic refinement during the early stages of brain development. In fact, the absence of *TREM2* resulted in impaired synapse elimination, accompanied by enhanced excitatory neurotransmission and reduced long-range functional connectivity [11]. Heterozygous variants of *TREM2* increase the risk of Alzheimer's disease (AD) and are involved in other neurodegenerative diseases (see [12] 2018 for review).

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Although doubts were raised on the expression of TREM2 in Peripheral Blood Mononuclear Cells (PBMC) [13], more recent evidence demonstrates that TREM2 mRNA is detectable in whole blood [14,15], and higher levels were observed in patients with AD compared to amnestic Mild Cognitive Impairment (MCI) patients and healthy subjects [15]. These data were further confirmed recently by Casati et al. [16], who found increased levels not only in AD but also in MCI subjects converted to AD at longitudinal follow up.

In this scenario, the aim of this study was to analyze PBMC profile of 164 inflammatory factors, including mainly cytokines and chemokines, in patients with NHD carrying *TREM2 Q33X* mutation as compared with heterozygous and wild type individuals.

2. Materials and methods

2.1. Subjects

Three patients with NHD carrying the Q33X mutations were included. Two belonged to the family 1, previously described by Bock et al. [17], whereas one was part of family 2, described recently by Ghezzi et al. [18]. Heterozygous parents (n = 4) were included as well and compared with 4 non-carriers (one from family 1 and three healthy volunteers). Details of all subjects are described in Table 1.

Informed consent was given by all subjects or their caregivers.

2.2. PBMC isolation

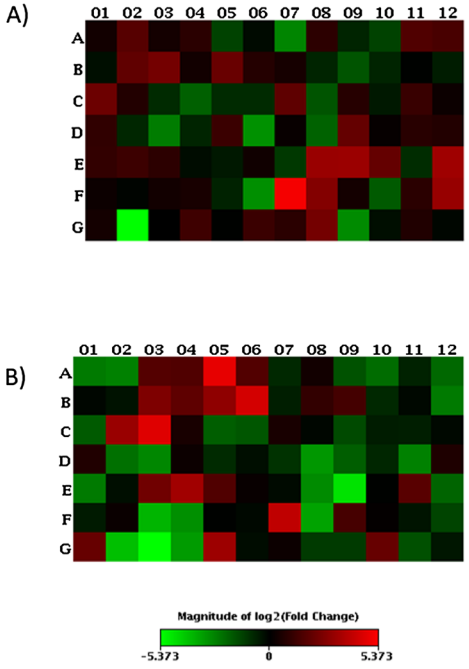
Ten mL of blood were collected in a BD Vacutainer CPT™ (1 mL NC, 2 mL Ficoll) as previously described [19]. PBMC were separated by gradient centrifugation and total RNA extracted with the single step acid phenol method, using Trizol (Invitrogen, Carlsbad, CA, USA). RNA integrity was measured by an Agilent 2100 Bioanalyzer showing an RNA Integrity Number that ranged from 8 to 10, whereas RNA concentration and purity was assessed with Nanodrop ND-1000 (Thermo Fisher Scientific).

Table 1
Characteristics of individuals studied and significant mRNA relative levels in PBMC, expressed as fold regulations over non-carriers.

	NHD (Q33X/Q33X)	Heterozygous (Q33X/wt)	Non-carriers (wt/wt)
n	3	4	4
Sex (M:F)	1:2	2:2	1:3
Age at onset (mean, ranges)	39 (37–41)	–	–
Age at sampling (mean, ranges)	43 (39–46)	71 (69–72)	51 (38–68)

Gene	mRNA relative expression (vs controls)			*P* value	Function
BMP-1	2.32*	1.74	1	0.01	↑ Bone formation and angiogenesis
TGFB3	1.51*	−1.07	1	0.02	Same family as BMP-1
CXCL5	−28.26*	1.84	1	0.01	↑ Angiogenesis and connective tissue remodeling
PPBP	−9.85*	−1.5	1	0.01	↑ Mitosis, glycolysis, synthesis of glycosaminoglycans
PF4V1	−41.44*	1.26	1	0.03	↓ Angiogenesis
IL-15	−2.25*	−2.05*	1	< 0.01	↑ Natural killer cell activation and proliferation
TNFSF3	−3.87*	−1.25	1	0.001	TNF family member

* Statistically significant.



layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ADIPOQ 1,20	**BMP1 * 2,32**	BMP2 1,22	BMP3 1,5	BMP4 -1,89	BMP5 -1,10	BMP6 -3,69	BMP7 1,53	CD40LG -1,41	CD70 -1,92	CNTF 2,18	CSF1 2,02
B	CSF2 -1,15	CSF3 2,55	FAM3B 3,09	FASLG 1,19	FIGF 2,75	GDF2 1,43	GDF5 1,24	GDF9 -1,42	IFNA1 -2,24	IFNA2 -1,41	IFNA4 -1,02	IFNA5 -1,32
C	IFNB1 2,88	IFNG 1,38	IL10 -1,50	IL11 -2,53	IL12A -1,51	IL12B -1,50	IL13 2,47	**IL15 * -2,25**	IL16 1,45	IL17A -1,25	IL17B 1,73	IL17C 1,12
D	IL18 1,60	IL19 -1,44	IL1A -3,33	IL1B -1,42	IL1RN 1,76	IL2 -4,11	IL20 1,09	IL21 -2,58	IL22 2,62	IL23A 1,05	IL24 1,48	IL25 1,40
E	IL27 1,61	IL3 1,81	IL4 1,54	IL5 -1,11	IL6 -1,26	IL7 1,17	CXCL8 -1,73	IL9 4,45	INHA 4,53	INHBA 2,64	LEFTY2 -1,54	LIF 4,76
F	LTA 1,12	LTB -1,04	MSTN 1,19	NODAL 1,26	OSM -1,39	PDGFA -4,06	SPP1 10,96	TGFA 3,59	TGFB1 1,22	TGFB2 -2,38	**TGFB3 * 1,51**	THPO 4,40
G	TNF 1,21	TNFRSF11B -11,92	TNFSF10 -1,00	TNFSF11 1,84	TNFSF12 -1,03	TNFSF13 1,76	TNFSF13B 1,51	TNFSF14 3,07	**TNFSF4 * -3,87**	TNFSF8 -1,14	TXLNA 1,37	VEGFA -1,06

layout	01	02	03	04	05	06	07	08	09	10	11	12
A	C5 -5,88	CSAR1 -6,61	ACKR2 3,38	CCL1 3,17	CCL11 29,71	CCL13 3,36	CCL14 -1,79	CCL15 1,3	CCL16 -3,32	CCL17 -4,81	CCL18 -1,66	CCL19 -4,62
B	CCL2 -1,10	CCL20 -1,29	CCL21 6,35	CCL22 3,93	CCL23 8,30	CCL24 22,17	CCL25 -1,53	CCL26 2,06	CCL27 2,69	CCL28 -1,81	CCL3 -1,12	CCL4 -6,03
C	CCL5 -3,73	CCL7 9,54	CCL8 26,14	CCR1 1,41	CCR10 -3,94	CCR2 -3,47	CCR3 1,42	CCR4 -1,09	CCR5 -2,98	CCR6 -1,52	CCR7 -1,56	CCR8 -1,11
D	CCR9 1,62	ACKR4 -5,07	CCRL2 -7,25	CKLF 1,21	CMKLR1 -1,84	CMTM1 -1,21	CMTM2 -2,09	CMTM3 -9,27	CMTM4 -3,90	CX3CL1 -1,73	CX3CR1 -6,61	CXCL1 1,57
E	CXCL10 -5,99	CXCL11 -1,21	CXCL12 5,12	CXCL13 10,51	CXCL14 3,22	CXCL16 1,13	CXCL2 -1,16	CXCL3 -7,79	**CXCL5 * -28,26**	CXCL6 -1,04	CXCL9 3,64	CXCR1 -4,36
F	CXCR2 -1,45	CXCR3 1,15	CXCR4 -14,30	CXCR5 -8,20	CXCR6 -1,03	ACKR3 -1,12	ACKR1 16,61	FPR1 -10,92	GPR17 2,81	H1F1A 1,07	IL16 -1,33	IL1B -2,68
G	IL4 4,39	CXCL8 -16,47	**PF4V1 * -41,44**	**PPBP * -9,85**	SLIT2 10,07	TLR2 -1,20	TLR4 1,19	TNF -2,35	TYMP -2,32	XCL1 4,44	XCL2 -3,19	XCR1 -1,39

Fig. 1. Left panels: relative heat maps of homozygous Q33X carriers versus controls. Green indicates down-regulation, red up-regulation. Right panels: layout of the arrays (A) Human Inflammatory Cytokines & Receptors PCR array (PAHS-011Z) and (B) Human Cytokines & Chemokines array (PAHS-150Z). Data are expressed as fold change (fold expression). Each square represents a gene; * indicates $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

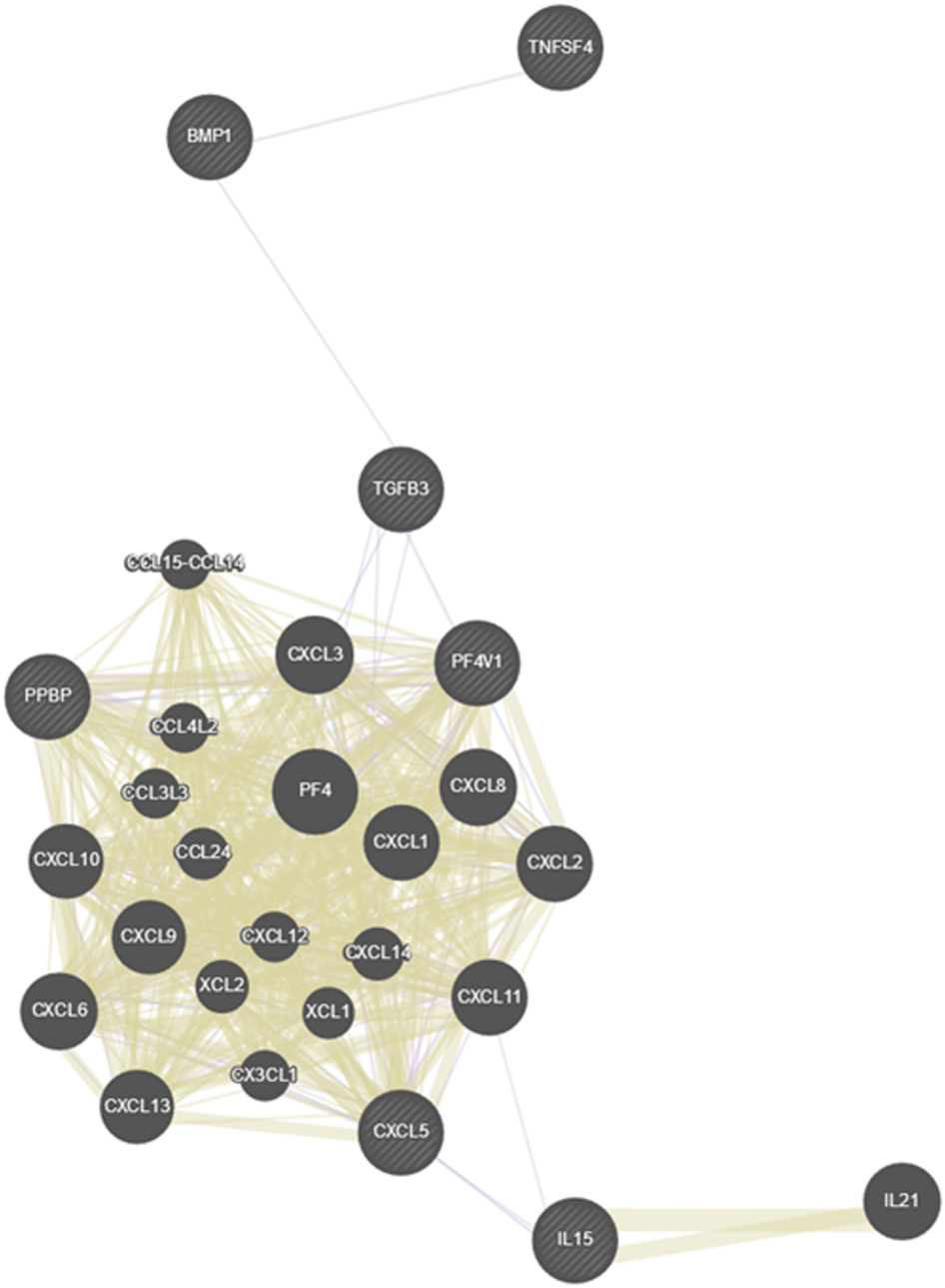


Fig. 2. Gene network analysis obtained by using the GeneMANIA algorithm. The seven de-regulated genes listed in Table 1 have been used to generate the map.

2.3. Screening of inflammatory molecules and related receptors by PCR arrays

RNA was retrotranscribed with RT2 First Strand Kit (SABiosciences, Frederick, MD, USA), according to the instruction of the manufacturer. For real time PCR experiments, the RT^2 Human Cytokines and Chemokines array and the RT^2 Human Inflammatory Cytokines & Receptors array (PAHS-150Z and PAHS-011Z, respectively, Qiagen, Hilden, Germany) were used for a total of 164 molecules analyzed. Real Time PCR were performed in an Applied Bio Systems 7500 FAST machine (Foster City, CA, USA). The arrays profile the expression of 164 inflammatory molecules, including cytokines, chemokines, and related receptors. These arrays included also five housekeeping genes (ACTB, B2M, G3PDH, HPRT1 and RPLP0) for the proper normalization of the data, mRNA reverse transcription control and a positive PCR control.

2.4. Statistical methods

The Qiagen PCR Array data analysis was based on $\Delta\Delta C_t$ method with normalization of the raw data to housekeeping genes. *P*-values were calculated based on a Student's *t*-test of the replicate 2 ∧(−DeltaCt) values for each gene in the control and NHD/carrier groups. Best hits for the validation analysis were chosen based on statistical significance ($P < 0.050$). Results from real-time PCR were calculated as Fold-Change [2∧(−Delta Delta Ct)], which was the normalized gene expression [2∧(−Delta Ct)] in the NHD group divided by the normalized gene expression [2∧(−Delta Ct)] in the control group [20].

3. Results

We evaluated, through Real time PCR using commercially available arrays, levels of 164 inflammatory transcripts, including cytokines,

chemokines, and related receptors, in PBMC from 3 NHD patients as compared with 4 heterozygous parents and 4 non-carriers.

Considering NHD patients versus non-carriers, several molecules related to bone formation and angiogenesis were altered (Table 1, Fig. 1): Bone Morphogenetic Protein (BMP)-1 mRNA levels were significantly increased in PBMC (2.32 fold-increase; $P = 0.01$), as were Transforming Growth Factor Beta (TGFB)3 levels (1.51 fold-increase; $P = 0.02$). A trend towards increased mRNA levels of BMP-2 and −3 were observed as well (1.22 and 1.5 fold-increase, $P = 0.44$ and 0.22, respectively). Conversely, CXCL5 and Pro Platelet Basic Protein (PPBP) were strongly downregulated in patients (−28.26, −9.85 fold-decrease over non-carriers, respectively, $P = 0.01$). A remarkable decrease in Platelet Factor 4 Variant 1 (PF4V1) was observed as well (−41.44, $P = 0.03$).

Among other inflammatory factors evaluated, Interleukin (IL)-15 and Tumor Necrosis Factor Superfamily Member (TNFSF)4 mRNA levels were decreased in NHD as compared with non-carriers (−2.25 and −3.87 fold-decrease, $P = 0.01$ and 0.001, respectively).

In heterozygous individuals, no significant differences were observed, apart from IL-15 mRNA levels, that were decreased at the same extent as NHD (−2.05 fold-decrease over non-carriers, $P = 0.002$, data not shown).

In an effort to delineate the pathways in which these genes could be involved, the GeneMANIA algorithm (http://www.genemania.org) [21] was utilized in a function prediction setting. As shown in the gene map of Fig. 2, new genes (circles in grey), that are functionally associated with those used to generate the map and listed in Table 1, were evidenced. From among these genes, the majority of genes identified encodes for chemokine, chemokine receptors or chemokine-motifs, thus representing additional promising candidates involved in NHD.

4. Discussion

Data shown here demonstrate that inflammatory factors play a role in the pathogenesis of NHD. In particular, a signature of NHD patients, consisting in altered expression levels in PBMC of BMP-1, TGFB3 (mildly increased), CXCL5, PPBP, PF4V1 (strongly decreased), IL-15, TNFSF4 (mildly decreased).

Both BMP-1 and TGFB3 were significantly up-regulated; both of them are members of the TGFβ superfamily of proteins, and they have been shown to regulate limb and digit formation, kidney development, cancer, angiogenesis and tissue fibrosis [22]. BMPs have been shown in pre-clinical models to stimulate bone formation and angiogenesis [22]. Notably, although not at a significant extent, also expression of other members of this superfamily, namely BMP-2 and -3, were increased. As NHD is associated with systemic bone cysts formation leading to fractures, the increased expression of BMPs, involved in bone formation, may represent an attempt to restore bone damage. Moreover, additional inflammatory transcripts playing a role in bone modelling and angiogenesis were strongly down-regulated: CXCL5, which promotes angiogenesis and remodelling of connective tissue; PPBP, which is chemoattractant for neutrophils and stimulates mitosis, glycolysis, and synthesis of hyaluronic acid and glycosaminoglycans; PF4V1, conversely displaying strong antiangiogenic functions. Notably, BMP-1 and TGFB3, were up-regulated in patients with NHD to a lesser extent than downregulated genes (i.e. 1.5/2 fold-increase compared with −10/−40 fold-decrease over controls), suggesting that in NHD patients there is a remarkable de-regulation of factors related to angiogenesis and bone repair. In addition, also IL-15, implicated in T cell regulation and natural killer cell activation and proliferation, was down-regulated, as well as TNFSF4, a member of the TNF family. Interestingly, IL-15 levels were found to be de-regulated in Cerebrospinal Fluid from patients with Frontotemporal dementia (FTD) [23,24], possibly playing a role in the occurrence of frontal symptoms in both FTD and NHD.

According to GeneMANIA algorithm, the majority of genes possibly belonging to pathways in which the seven de-regulated genes identified here are involved in, encodes for chemokine, chemokine receptors or chemokine-motifs, thus representing additional promising candidates involved in NHD. We hypothesized that these genes had not been identified in our study due to the small number of cases analysed or possibly to a low abundance of mRNA in PBMC.

Limitations of this study include the small number of patients considered and the lack of corresponding protein analysis.

5. Conclusion

In conclusion, we identified a signature in PBMC from patients with NHD consisting of strongly decreased mRNA levels of CXCL5, PPBP, PF4V1, mildly decreased IL-15 and TNFSF4 and mildly increased BMP-1 and TGFB3, possibly playing a role in bone modelling and the occurrence of dementia in these patients. Regarding the latter, there is evidence of an involvement of inflammation in FTD and AD, but larger studies would be needed to confirm a causal role between an imbalance of specific inflammatory factors and the occurrence of dementia in patients with NHD.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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