NKT-like Cells are Expanded in Solid Tumour Patients

Zvýšený počet NKT-like buněk u pacientů se solidními nádory

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Summary
CD3+ CD56+ NKT-like cells have been shown to produce substantial amounts of pro-inflammatory cytokines and to mediate lysis of malignant cells. Using flow cytometry, we evaluated the absolute NKT-like cell count in peripheral blood from individuals in a reference population and the median number was 0.085 × 10^9/l. The average number of NKT-like cells in patients with disseminated cancer was 2.65 fold higher than in the reference population. The number of CD3+ CD56+ cells in solid tumour patients who achieved complete remission was comparable to the reference population. In breast cancer patients with initially (prior to therapy) increased number of NKT-like cells, we observed a trend toward longer disease-free survival. Thus we conclude that CD3+ CD56+ NKT-like cells have potential to suppress tumour evasion and are expanded in peripheral blood of some epithelial tumour patients.

Key words
NKT-like cells – cancer – CD3+ CD56+ cells – flow cytometry – breast neoplasms

Souhrn
CD3+ CD56+ NKT-like buňky produkují značné množství prozánětlivých cytokinů a mají schopnost zprostředkovat lýzu maligních buněk. Za použití průtokové cytometrie jsme hodnotili absolutní počet NKT-like buněk v periferní krvi jedinců z referenční populace, přičemž střední hodnota zde byla 0,085 × 10^9/l. Průměrný počet NKT-like buněk u pacientů s diseminovaným nádorovým onemocněním byl 2,65krát vyšší než v referenční populaci. Počet CD3+ CD56+ buněk u pacientů se solidní malignitou, kteří dosáhli kompletní remise onemocnění, byl srovnatelný s referenční populací. U pacientek s karcinomem prsu s iniciálně (před zahájením terapie) zvýšeným počtem NKT-like jsme pozorovali trend k prodlouženému přežití bez progrese onemocnění. Ze studie vyplývá, že CD3+ CD56+ NKT-like buňky mají potenciál potlačovat rozvoj nádoru a jejich počet je zvýšen u určitých typů epiteliálních nádorů.

Klíčová slova
NKT-like buňky – rakovina – CD3+ CD56+ buňky – průtoková cytometrie – nádory prsu

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Introduction

Natural killer T (NKT) cells are unusual lymphocytes coexpressing some NK markers and possessing self-reactivity and capacity to secrete large quantities of cytokines, such as IFN-γ in mice [1,2]. NKT cells represent a heterogeneous group that consists of Type I cells (Classical NKT cells), Type II cells (Non-classical NKT cells) and NKT-like cells (CD1d-independent NK1.1+ T cells) reviewed in [3]. CD3+CD56+ NKT-like cells represent a minor population in peripheral blood [4]. These cells have been shown to mediate lysis of malignant cells and to produce substantial amounts of cytokines [5–8].

We evaluated the NKT-like cell absolute count in our regional reference population and in cancer patients. We aimed to investigate whether an initially increased NKT-like cell count in breast cancer patients favours disease-free survival.

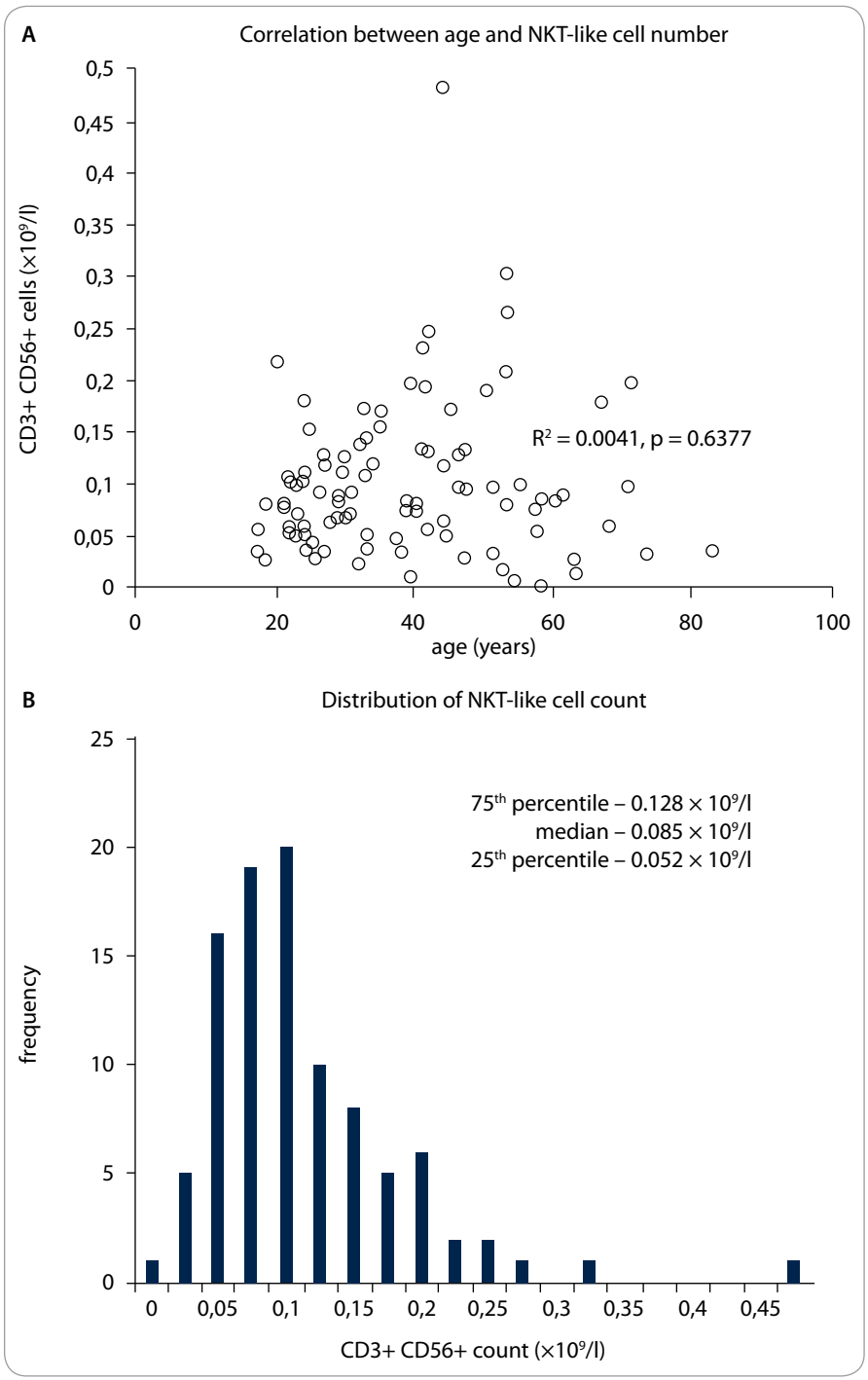

Fig. 1. NKT-like cells in common population. A. Correlation between age and NKT-like cells number. Each measurement is shown as an open circle. The line represents linear flowline, R is correlation coefficient, p is statistical significance. B. Histogram showing the distribution of NKT-like cell counts. Values for 75th, 50th and 25th percentile are shown.

Material and Methods

Patients and Controls

NKT-like cells were analysed in cancer patients, hospital staff and clients of preventive care clinic of Masaryk Memorial Cancer Institute. Evaluated individuals signed the informed consent. The reference/common population consisted of cancer-free controls: 56 individuals from the hospital staff and 41 clients of preventive care. Cancer patients: the group of disseminated cancers consisted of 20 cases of metastatic breast tumours, 16 metastatic colorectal cancers, 14 disseminated kidney tumours, 9 cases of generalised malignant melanoma, 5 pancreatic cancers, 3 cases of metastatic prostate cancer, 3 disseminated testicular tumours, 1 case of metastatic hepatocellular carcinoma and 1 case of penile cancer. The group of patients in remission consisted of 16 cases of breast tumours, 6 colorectal cancers, 3 kidney tumours, 13 cases of malignant melanoma and 3 testicular tumours. Breast cancer evaluation: 31 patients diagnosed for breast cancer between March 2004 and September 2005 were evaluated for NKT-like cell count prior to treatment. The group consisted of patients with various stage of disease; 11 of them with distant metastases. The follow-up for disease progression was performed in August 2012.

Immunophenotype Analyses

Peripheral blood was collected into EDTA test tubes (Sarstedt) and processed within 4 hours after blood withdrawal. Complete blood count with white blood cell differential was measured using Sysmex XE 5000 hematologic analyser. Cell staining for flow cytometry was performed with 50 µl of full blood

with the following monoclonal antibodies: CD3-FITC (clone UCHT1, 10 µl), CD8-PE (B9.11, 5 µl), CD56-PC5 (N901, 10 µl), CD4-PC7 (SFCI12T4D11, 5 µl), purchased from Beckman Coulter. After a 15 min incubation in the dark at the room temperature, red blood cells were lysed with 600 µl of VersaLyse (Beckman Coulter) for 15 min and flow cytometric analysis was performed immediately using FC500 instrument (Beckman Coulter). Lymphocytes were gated based on SS/FS properties and the correction was performed using CD3 measured in another test tube together with CD45. NKT-like cells were assessed as CD3+ CD56+ cells and their absolute count was calculated using the number of lymphocytes measured by hematologic analyser.

Statistical Analyses

Survival differences between NKT-like low and NKT-like high population of patients were compared using log-rank test. NKT-like low group consisted of breast cancer patients with NKT-like cell count lower than 25th percentile of the normal population count, while NKT-like high group of the population higher than 75th percentile of common population.

Differences of NKT-like cell counts between two populations were tested using two-sided Mann-Whitney two sample tests. In case of multiple hypotheses testing, p-values were adjusted by Benjamini-Hochberg correction and significance level was set to 10% correlation between age and NKT-like cell count was calculated using Spearman's rank coefficient.

Results

NKT-like Cells in Reference/Common Population

We enumerated the number of NKT-like cells in peripheral blood of 97 individuals from the reference/common population. The group consisted of 70 women and 27 men. We did not observe statistically significant difference in the CD3+ CD56+ count between men and women (p = 0.089) and the NKT-like cell number was age-independent (Fig. 1A, p = 0.6377). The median number of NKT-like cells in the reference population was 0.085 × 10^9/l (Fig. 1B).

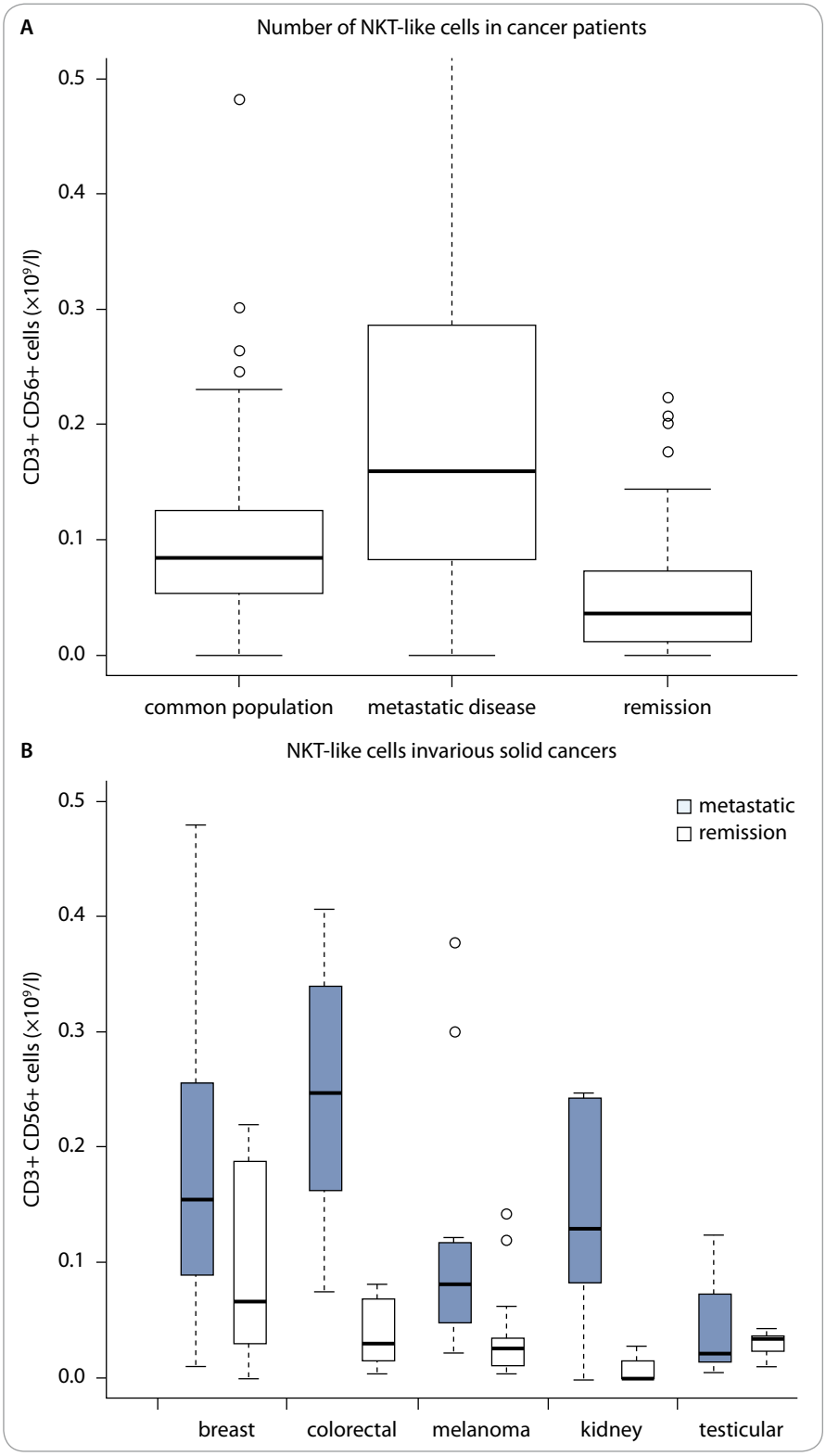

Fig. 2. Distribution of NKT-like cells in cancer patients. Box and whisker plots represent median (horizontal bar), interquartile range (box), 1.5-times interquartile range (whiskers) and outliers (points). For the purpose of readability, the y-axes represent a zoom from the whole scale range (0–1.9133). Figures in original scale are available in supplementary material. A. Comparison of NKT-like cell number in common population, cancer patients with metastatic disease and in cancer patients with disease in remission. B. Differences of NKT-like cells counts between remission and metastatic disease in various cancer diagnoses.

Tab. 1. Summary of tumour and clinical information and number of NKT-like cells in breast cancer patients.

Case	Clinical stage	Tissue type / grade	Months to progression /months of disease-free survival	NKT-like cell × 10^9/l
1	ypT1c, pN1b1, pMx	invasive ductal / G3	94+	0.150
2	T2-3, N0, Mx	invasive lobular / na	37	0.069
3	T3-4, N1, M1	invasive ductal / G1	12	0.005
4	T4b + d, N1, M0	invasive ductal / G3	100+	1.250
5	T3, N1b, M1	invasive NOS / G3	3	0.100
6	T4b, N1b, M0	invasive ductal / na	7	0.128
7	T3, Nx, M1	bilateral: 1/spinocellular, 2/ invasive lobular / G1–2	88+	0.051
8	T4d N1 M0	trabecular / na	19	0.021
9	T4b N1 M1	invasive ductal / G3	95+	0.075
10	T2 N1 M0	invasive ductal / G3	91+	0.077
11	T2–3 N1 M0	invasive ductal / G2–3	94+	0.050
12	T2 N1 M0	invasive ductal / G3	39	0.129
13	T4b N1b M0	invasive ductal G2–3	26	0.079
14	pT2 pN1b4 M0	comedo-type ductal / G3	16	0.045
15	T1b N0 Mx	invasive ductal / na	99+	0.064
16	pT1 N1 M1	invasive ductal / na	46	0.095
17	pT1b Nx M0	invasive ductal / G1	93+	0.024
18	pT2 pN0 M0	invasive papillary / G2	100+	0.057
19	T4b N2 M1	invasive ductal / G1–2	5	0.012
20	T4b N2 M1	na / na	10	0.113
21	T2N1M0	invasive lobular / G3	84+	0.141
22	T2–3 N1b M0	invasive tubular / na	100+	0.012
23	T4d N1 M0	invasive ductal / G3	94+	0.192
24	T4c N1 M1	invasive ductal / G2	2	0.011
25	T2 Nlb M1	invasive ductal / G1–2	6	0.060
26	T2 N1b M0	invasive ductal / G2–3	90+	0.023
27	pT4b Nlb3 M0	invasive ductal / G2	93+	0.059
28	ypT4b N0 M0	invasive squamous / G3	83+	0.090
29	T4b N1 M1	invasive ductal / na	24	0.109
30	T2 N1 MO	invasive ductal / G2	88+	0.057
31	T2 N1 M1	invasive ductal / G3	7	0.029

„+" in the time-to-progression column indicates duration of remission.

NKT-like Cells in Cancer Patients

The number of NKT-like cells was measured in patients with disseminated cancer and the disease in remission. The average number of NKT-like cells in patients with disseminated cancer was 2.65 fold higher than in the reference population (Fig. 2A, p = 0.00003365). The average number of NKT-like cells in the remission group was on the other hand 1.25 fold lower compared to the common population (p = 0.00005043). Within the metastatic group, a non-significantly increased number of NKT-like cells was observed in colorectal carcinomas compared to malignant melanoma (FDR = 0.0722) and to testicular tumours (FDR = 0.0722) (Fig. 2B). When comparing disseminated and remission group within one diagnosis, significantly higher NKT-like count was observed in disseminated tumours in all diagnoses, except testicular cancer (FDR of 0.0499; 0.00027; 0.0452 and 0.0452 for breast, colorectal, melanoma and kidney cancer, respectively, Fig. 2B).

NKT-like Cell in Breast Cancer Patients

The number of NKT-like cells was evaluated in 31 patients with breast cancer between diagnosis and treatment. Subsequently, the follow-up for disease-free survival was performed. During the 7-year follow-up, 16 patients achieved complete remission of the disease and in 15 cases the disease progressed (Tab. 1). There was a trend towards increased frequency of progression in the NKT-like low group of patients, but it was not significant (Fig. 3).

Discussion

NKT-like cells represent a subset of T-lymphocytes expressing some natural killer cell receptors. These cells are considered to be associated with effector-memory and effector T-lymphocyte subpopulations and thus their count to be increased with age [9]. In our study we have not observed age-dependent change in NKT-like cell counts, however this discrepancy might be attributed to the lack of elderly individuals and centenarians in our study population.

The expansion of CD3+CD56+ NKT-like cells in cancer patients with active disease but not with disease in remission may reflect the presence of the active malignancy leading to stimulation of the cytotoxic arm of the immune response including NKT-like cells. This finding is in line with the observation that CD8+ NKT-like cells were expanded in tumour-bearing C57BL/6 mice [10]. On the other hand, we did not observe a correlation between CEA or CA15-3 tumour marker levels and the NKT-like cell count (data not shown) and within the group of breast patients prior to anti-cancer therapy there was no difference in NKT-like cells count in initially metastatic vs localised disease.

Concerning the immunophenotype, the majority of NKT-like cells in our study were CD16- and expression of CD8 predominated CD4 expression (data not shown). CD8+ NKT-like cells have been shown to produce large amounts of IL-10 and IFN-γ [11] and to lack the production of IL-4 [12]. Anti-tumour activity of NKT-like cell could be mediated by

IFN-γ without IL-4 resulting into pro-inflammatory TH1 immune response. Anti-tumour activity of NKT-like cells could be further attributed to several compounds upregulated by NKT-like cells and involved in cytotoxicity, such as perforin, granzymes and TNF family proteins [11]. It was shown that NKT-like cells are an important source of pro-inflammatory cytokines, IFN-γ, TNF-α, IL-2 and IL-17, and granzymes and thus this cell subpopulation might be involved in lung transplant pathology [13]. Our preliminary data from disease-free survival in NKT-like-high breast cancer patients and the pro-inflammatory cytokine production suggest that NKT-like cells suppress solid tumour growth. Similarly, a protective role of NKT-like cells has been described for chronic lymphocytic leukaemia [14].

Focusing on various tumour origin, increased count of NKT-like cells have been observed in breast, colorectal and kidney tumour cases, but not in testicular tumours and malignant melanoma, suggesting that NKT-like cell expansion occurs predominantly in epithelial tumours. Malignant melanoma cells often express molecules suppressing anti-tumour activity of tumour infiltrating lymphocytes, e.g. galectin-3 and galectin-1 [15,16]. Other potent tools of melanoma cells to impair NKT-like cytotoxicity are expression of soluble MHC class I chain-related molecules [17] and expression of NKG2D ligands [18,19]. Absent stimulation of NKT-like cells in testicular tumour might be related to the fact that testes represent an immune privileged site and tumours arising from this tissue are often not accessible by immune cell response.

In conclusion, CD3+ CD56+ NKT-like cells with potential to suppress tumour evasion are expanded in peripheral blood of some epithelial tumour patients.

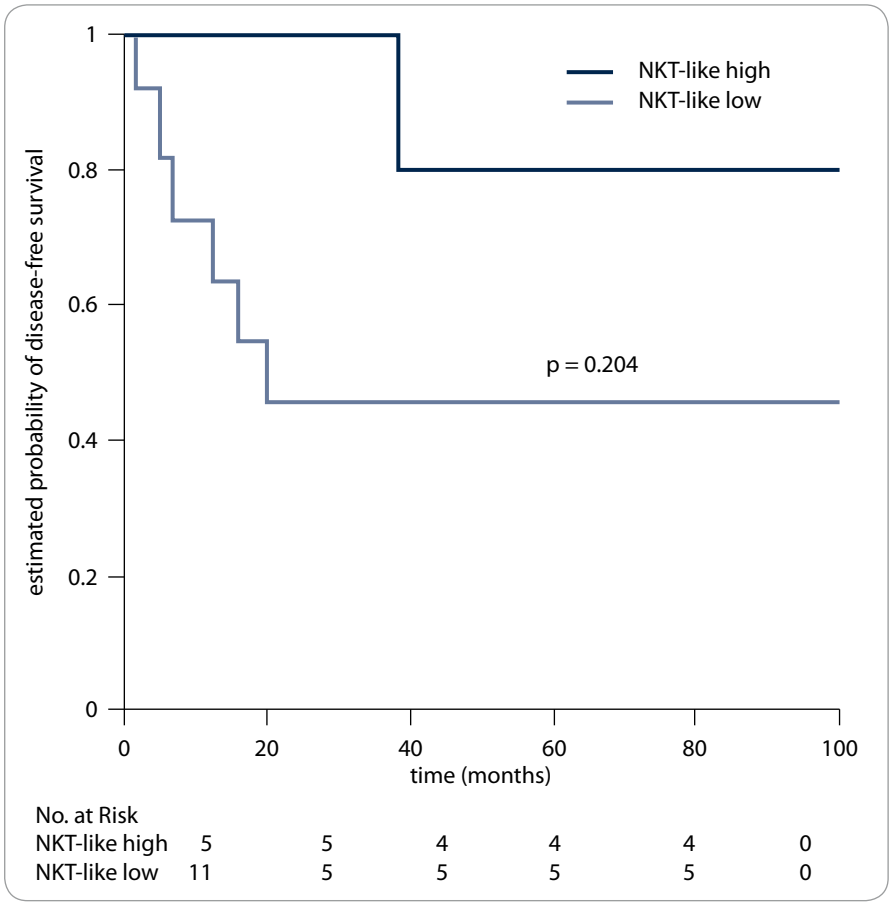

Fig. 3. Disease-free survival Kaplan-Meier curves in NKT-like low and NKT-like high breast cancer patients. NKT-like low group consists of patients with NKT-like cell number lower that 25th percentile of common population and NKT-like high group represents breast cancer patients with NKT-like above 75th percentile of common population.

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