Inhibition of chemerin/CMKLR1 axis in neuroblastoma cells reduces clonogenicity and cell viability in vitro and impairs tumor growth in vivo

SUPPLEMENTARY MATERIALS

Supplementary Figure 1: Expression data was analyzed using the R2 database http://r2.amc.nl. Kaplan-Meier survival estimates were used to evaluate the prognostic value of RARRES2 (chemerin) (A, B) and CCRL2 (D, E) expression in two patient data sets (A and D: Versteeg n=88; B and E: Seeger n=102). The Kaplan-Meier scanning tool was used to determine the RARRES2 (chemerin) and CCRL2 mRNA expression in neuroblastoma. All expression data were scanned to find the most optimal cut-off between high and low gene expression and the log-rank test that gave the lowest p-value was calculated to search for significant differences between tumor samples expressing high and low RARRES2 (chemerin) and CCRL2 mRNA levels, respectively. The expression of RARRES2 (chemerin) (C) and CCRL2 (F) was compared between neural crest (Etchevers n=5), benign neurofibroma (Miller n=86) and 4 neuroblastoma cohorts (cohort 1: Versteeg n=88, cohort 2: Delattre n=64, cohort 3: Hiyama n=51, cohort 4: Lastowska n=30).
Supplementary Figure 2: **CMKLR1** (A), **RARRES2** (B) and **GPR1** (C) expression was analyzed using the Russel neuroblastoma cell line panel (transcript) in the R2 database http://r2.amc.nl. Cell lines used in this study were marked with an asterisk. The expression of **CMKLR1** and **GPR1** was compared to the previously in neuroblastoma cell lines described receptors **FPR1**, **IL6R** and **PDGFRA** (D). Chemerin expression was compared to **CCL2**, **CCL5** and **VEGFA** (E).
Supplementary Figure 3: Immunofluorescence CMKLR1 staining using SK-N-AS, SK-N-BE(2), SH-SY5Y and SK-N-SH cells and two different antibodies: STJ92262 from St. John’s laboratory (A) and LS-B12924 from LSBio (B). The used secondary antibody was Goat anti-Rabbit IgG (H+L), Alexa Fluor 488 (A-11008, Thermo Fisher Scientific) and the nuclei were stained with Hoechst 33342 (ImmunoChemistry Technologies). Isotype control staining for IF and IHC. (C) SH-SY5Y cells were incubated with rabbit isotype antibody instead of primary antibody. Immunoperoxidase labeled tissue sections where the primary antibodies were replaced with rabbit (D) or mouse (E) isotype antibodies. Neuroblastoma tissue sections were incubated with mouse and goat isotype antibodies prior to fluorescence staining (F).
Supplementary Figure 4: The tumor growth rate in neuroblastoma bearing mice was significantly lower in the α-NETA pre-treatment group compared to the control group (one-way ANOVA P=0.0061, Bonferroni post-test, control vs. pre-treatment P=0.049). Growth rate in the different treatment groups was illustrated as the slope from curves over individual mice log tumor volume versus time. Goodness of curve fitting was assessed with R^2 (R^2 mean 0.97; range 0.875-0.999). The line represents mean slope in each treatment group (A). Individual tumor growth in (B) control mice (daily s.c. injections with 10% Captisol®), (C) pre-treatment group (daily s.c. injections with 10 mg/kg α-NETA from the day after tumor cell injection and 20 mg/kg when the tumor reached 0.15ml) and (D) treatment group (daily s.c. injections with 20 mg/kg α-NETA after the tumor reached 0.15ml).
Supplementary Figure 5: Expression data was analyzed using the R2 database http://r2.amc.nl. The Asgharzadeh neuroblastoma cohort n=249 was used to correlate the expression of previously identified TAM markers (CD33, CD14, IL6R, IL10, FCGR3) to CMKLR1 expression.
Supplementary Figure 6: Immunoperoxidase staining of the macrophage marker CD68 using M0814 from Dako (A, B) and CMKLRI using STJ92262 from St. John’s laboratory (C) in neuroblastoma primary tumor tissue demonstrates while the majority of cells in the TME are positive for CMKLRI only few are positive for CD68. Double immunofluorescence labeling (D, E) of CMKLRI (green) and CD68 (red) confirms this observation. The used secondary antibodies were Goat anti-Rabbit IgG (H+L), Alexa Fluor 488 (A-11008, Thermo Fisher Scientific) and Donkey anti-Mouse IgG (H+L), Alexa Fluor 594 (A-21203, Thermo Fisher Scientific). The nuclei were stained with DAPI (blue). Isotype control staining is displayed in panel (F).
Supplementary Figure 7: SK-N-AS cells were transfected with sgRNA/Cas9 targeting all variants for human CMKLR1 or scrambled sgRNA control plasmids (GeneCopoeia, Inc., Rockville, USA) using jetPRIME® transfection reagent (Polyplus-transfection®, Illkirch, France) according to the provided manual. Forty-eight hours post transfection the cells were seeded as single cells into 96-well plates. After clonal expansion, the clones were screened for CMKLR1 expression using western blot and RT-PCR. Western blot of SK-N-AS wild-type (wt), scramble control (scr control) and CMKLR1 knockdown (CMKLR1 kd) using two different antibodies targeting CMKLR1 (A, STJ92262, St. John’s laboratory and B, ab64881, abcam) showing a clear reduction in CMKLR1 protein in the knockdown clone. RT-PCR analysis demonstrating a downregulation of CMKLR1 transcripts using two different primer sets (C). In clonogenicity assays (D-H) slower growth and the formation of indistinct colonies was observed in CMKLR1 knockdown cells. While small but distinct colonies were observed 6 days after cell seeding in the wild-type (D) and scramble control (E) the CMKLR1 knock down cells multiplied slower and were only loosely connected (F). After completion of the assays macroscopic colonies were observed in the scramble control (G) but not the CMKLR1 knockdown cells (H).