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Title

Hypoxic adaptation of leukemic cells infiltrating the CNS affords a therapeutic strategy targeting VEGF

Running Title: Hypoxic adaptation of leukemic cells in the CNS

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The central nervous system (CNS) is a key site of extra-medullary disease in pediatric acute lymphoblastic leukemia (ALL), [1] and prior to the development of contemporary risk adapted treatment strategies, CNS involvement was inevitable in most cases.[1] However, the biology of those leukemic cells that reside in this site is poorly understood and indeed, the extent to which genetic, transcriptional, and phenotypic variegation exists between leukemic cells located in different organs such as the CNS is unknown. To address these questions, we have undertaken a detailed analysis of primary B-ALL cells isolated from the CNS and bone marrows (BM) both from affected children and mouse xenograft recipients.

Leukemic cells isolated from the BM of patients presenting with B-ALL were transplanted into recipient mice by tail-vein injection (supplemental Table 1).[2] We confirmed BM engraftment in recipients by flow cytometry, and using both histological and radiological techniques we could demonstrate that they had patterns of CNS involvement mimicking those seen in patients with CNS disease (supplemental Figure 1,2). B-ALL cells have an inherent capacity to infiltrate the CNS,[3] and we observed that by 6 months recipient mice had CNS disease, regardless of whether the patients whose leukemic cells they received had CNS disease at the time the cells were donated for research. Thus this xenograft model faithfully recapitulates the natural clinical history of untreated B-ALL, and we used such mice, which had been allowed to develop CNS disease for our studies.

We compared gene expression profiles of leukemic cells isolated from the CNS ("CNS-derived leukemic cells") and BM ("BM-derived leukemic cells") of recipient mice (supplemental Table 2). Initially, we screened BM-derived and CNS-derived leukemic cells from recipients of 4 different ALLs (ALL#1-4) using microarrays. Gene set enrichment analysis (GSEA) showed that in CNS-derived leukemic cells, cell cycle- and oxidative phosphorylation-related gene sets were downregulated (supplemental Figure 3A; supplemental Table 3).

We next asked whether these differences were evident in the leukemic cells in the BM and cerebrospinal fluid (CSF) from 3 patients with B-ALL who had both CNS and BM involvement (ALL#6, #7, and #8). Despite containing varying numbers of leukemic cells, we found that cell cycle- and oxidative phosphorylation-related genes in the CNS-derived leukemic cells were similarly downregulated (supplemental Figure 3B; supplemental Table 4).

During the course of this work, a patient (ALL#9) presented with B-ALL and radiological evidence of leukemic meningitis. We transplanted this patient's BM sample into xenograft recipients, in order to study the physiology and transcriptional profiles of the BM- and CNS-derived leukemic cells. Leukemic cells from the two sites had similar surface immunophenotypes(supplemental Figure 4). However fewer of the CNS cells were in the S/G2/M phase of the cell cycle (Figure 1A-B), and consistent with this, a lower proportion of leukemic cells in CNS were actively proliferating as assessed by Ki67 staining (Figure 1C; supplemental Figure 5). In addition, CNS-derived leukemic cells consumed less oxygen in *in vitro* assays, indicating diminished mitochondrial activity (Figure 1D). These observations supported the transcriptional observations made in our initial microarray screen and the 3 primary patient samples.

We then asked whether these physiological differences could be explained by any genetic differences between CNS- and BM-derived leukemic cells in the xenograft recipients of ALL#9. We used targeted-capture deep sequencing of the diagnostic sample, and of CNS- and BM-derived leukemic cells from 2 recipient mice to compare genomic mutations and copy-number abnormalities in cells isolated from the two microenvironments, and we found no obvious differences between them (supplemental Figure 6; supplemental Table 5). Therefore we analyzed their transcriptomes to identify a gene expression signature specific to the CNS-derived cells. We compared leukemic cells from the BM, CNS and spleens from 5 recipients of BM from ALL#9 (supplemental Figure 7). Gene sets uniquely up-regulated in CNS-derived leukemic cells included those associated with hypoxia and glycolysis (Figure 1E), and as expected, cell cycle gene sets were down-regulated compared to BM-derived cells (supplemental Table 6). We observed no obvious expression changes in gene sets encoding cell adhesion molecules. Interestingly, hypoxic genes were also upregulated in CNS leukemic cells isolated from 3 patients (supplemental Figure 3C). Increased expression of the glycolytic enzymes hexokinase 2 (*HK2*) and pyruvate dehydrogenase Kinase1 (*PDK1*), which are known to be up-regulated in response to hypoxia,[4] was confirmed by RQPCR (supplemental Figure 8). Consistent with these differences in gene expression, we found that CNS-derived leukemic cells had much higher levels of glycolytic intermediates compared to those derived from the BM (Figure 1F). Our results indicate that CNS-derived leukemic cells are not genetically

distinct, but are both transcriptionally and physiologically adapted to hypoxic conditions.

VEGFA (a hypoxia responsive gene[5]) was upregulated in both CNS-derived leukemic cells (supplemental Table 7, Figure 1G) and in also primary leukemic cells isolated from the CSF of children with CNS involvement (Figure 1H). We confirmed this by RQPCR in the CNS leukemic cells of xenograft recipients (Figure 1I), and also observed that they express high levels of the VEGFA receptor (*FLT*-1/VEGFR1) (supplemental Figure 8). FLT-1 is commonly expressed in childhood ALL[6,7] and VEGFA/FLT-1 signaling promotes migration and survival of leukemic cells in vivo and vitro. [6,8]

Bevacizumab is a VEGFA-neutralizing antibody used in the treatment of several non-hematological cancers, and we tested its impact on mice with CNS involvement in our xenograft model. Recipients with equivalent levels of BM engraftment (Figure 2A), and therefore presumably similar levels of CNS involvement, were given intraperitoneal injections of either Bevacizumab or saline. The CNS leukemic burden was significantly reduced in mice treated with Bevacizumab (Figure 2B,C), and TUNEL staining demonstrated increased apoptosis in CNS-derived leukemic cells (Figure 2D, Supplemental Figure 9A). Although some TUNEL positive apoptotic cells were seen in BM in Bevacizumab recipients (Supplemental Figure 9B), its impact on BM disease burden was less marked (Figure 2A, B).

In conclusion, we have shown that B-ALL cells that infiltrate the CNS are more quiescent, consume less oxygen and have greater glycolytic activity than those residing in the BM, showing features of enhanced adaptation to hypoxia. This is consistent with the CNS being a harsher, more hypoxic and nutrient-poorer microenvironment than the BM.[9] We have previously reported that the small numbers of residual leukemic cells that remain in the BM of B-ALL patients who have undergone treatment are profoundly quiescent and consequently resistant to conventional chemotherapy, and we have proposed that these cells make a contribution to relapse.[10] Combined with the observations that hypoxic environment confers resistance to chemotherapy[11] and is associated with minimal residual disease of leukemia,[12] our data correlates with the clinical observation that residual CNS disease can drive relapse of B-ALL. It is possible that the ‘hypoxic adaptation’ we see in CNS-infiltrating childhood B-ALL is also found in other types of CNS-infiltrating hematological malignancies including ‘high

risk' groups with CNS involvement.[1] And, one interesting possibility is that VEGFA may be expressed in the limited leukemic cells adapted to hypoxic microenvironments in the BM, and Bevacizumab could target them. Although the number of patients analyzed is limited, and larger studies are required, we suggest that in the light of these data and the wide clinical experience with Bevacizumab together with its favorable toxicity profile warrant its inclusion in future clinical trials for B-ALL with CNS involvement.

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Authorship

Contributions: I.K., Y.N., A.N., and H.O. designed and performed experiments and analyzed data. M.M. performed animal experiments. M.N., D.W., and A.W. performed bioinformatics analyses and discussion. A.U.A. and T.M. performed pathological analysis and discussion. K.Y., H.U., M.N., A.W., Y.S., Y.S., and M.S. analyzed genetic materials. M.M., R.G., K.U., K.W., K.K., and S.A. provided clinical samples. T.E., T.N., T.H., M.K.S., S.O., and M.S. supervised the project and experimental design. I.K., R.G., and T.E. wrote the paper with input from Y.N., A.N., H.O., M.N., A.W., K.Y., D.W., and T.M.

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Figure Legends

Figure 1. Leukemic cells in the CNS show adaptation to hypoxia, such as reduced proliferation and oxidative phosphorylation, increased glycolysis and upregulation of *VEGFA*.

(A) Representative cell cycle analysis of DAPI-stained CNS- and BM- derived leukemic cells from xenograft recipients of ALL#9 leukemic cells. The fraction of cells in S/G2/M (8.6% vs 26.0%) is indicated. (B) Mean percentages of CNS- and BM-derived leukemic cells that are in S/G2/M from 5 separate recipients of ALL#9 leukemic cells. (C) Immunohistochemical analysis of leukemic cell proliferation in BM and brains from xenograft recipients of ALL#9 leukemic cells. The percentage of hCD19 +Ki67+ cells out of hCD19+ cells are indicated. *n*=3, biological replicates. (D) Mean oxygen consumption rates (RFU/hr) in CNS- and BM-derived leukemic cells from recipients of ALL#9 leukemic cells. *n*=3 technical replicates and data are representative of 2 independent experiments. Data show mean ± SEM, analysed with 2- sided, paired student *t*-test: *$P < 0.05$. (E) GSEA of RNA-seq data illustrating enrichment of hypoxia (NES=2.34, FDR=0) and glycolysis (NES=2.25, FDR=0) associated genes in CNS-derived leukemic cells from recipients of ALL#9 cells. (F) Heat map indicating levels of glycolytic metabolites in paired CNS- and BM-derived leukemic cells from 4 xenograft recipients (1 – 4) of ALL #9 leukemic cells measured by mass spectrometry. G1P, glucose 1 phosphate. G6P, glucose 6 phosphate. F6P, fructose 6 phosphate. 3PG, 3 phosphoglycerate, 2PG, 2 phosphoglycerate. Red, high concentration. Green, low concentration. (G) Quantification of *VEGFA* (probe ID: A_23_P70398) transcripts in CNS- and BM-derived leukemic cells from xenograft recipients of ALL #1-4 leukemic cells with microarray. ALL#3 contains data from 2

mice (3-1, 3-2). (H) Quantification of *VEGFA* in RNA-seq from primary tissues of ALL #6-8. (I) *VEGFA* expression by RQPCR in CNS- and BM-derived leukemic cells from recipients of ALL #9 leukemic cells, n=4 biological replicates. Data show mean± SEM, analysed with 2-sided, paired student t-test: *$P < 0.05$.

Figure 2. Effect of Bevacizumab on the leukemic cell burden in the CNS of xenograft recipients.

Leukemic mice showing >90% engraftment in the BM received intraperitoneal Bevacizumab or normal saline (NS). (A) Mean burden of leukemic cells in the BM of each group at the start and the end of treatment. (B) Mean burden of leukemic cells in the BM and CNS of each group at the end of treatment showing significant lower leukemic cells in the CNS of Bevacizumab treated mice. Data show mean and analysed with 2-sided, unpaired student t-test: ***$P < 0.001$. (C) Representative immunohistochemical staining for human CD19 in tissue sections from the CNS of NS and Bevacizumab treated mice. Thick blue arrows mark leukemic cells. Scale bar 250um.(D) The percentage of TUNEL positive leukemic cells in the CNS were counted from three distinct areas of the each section of two control mice and three bevacizumab treated mice. Graf shows the percentage of TUNEL positive cells in the CNS.

Figure 1

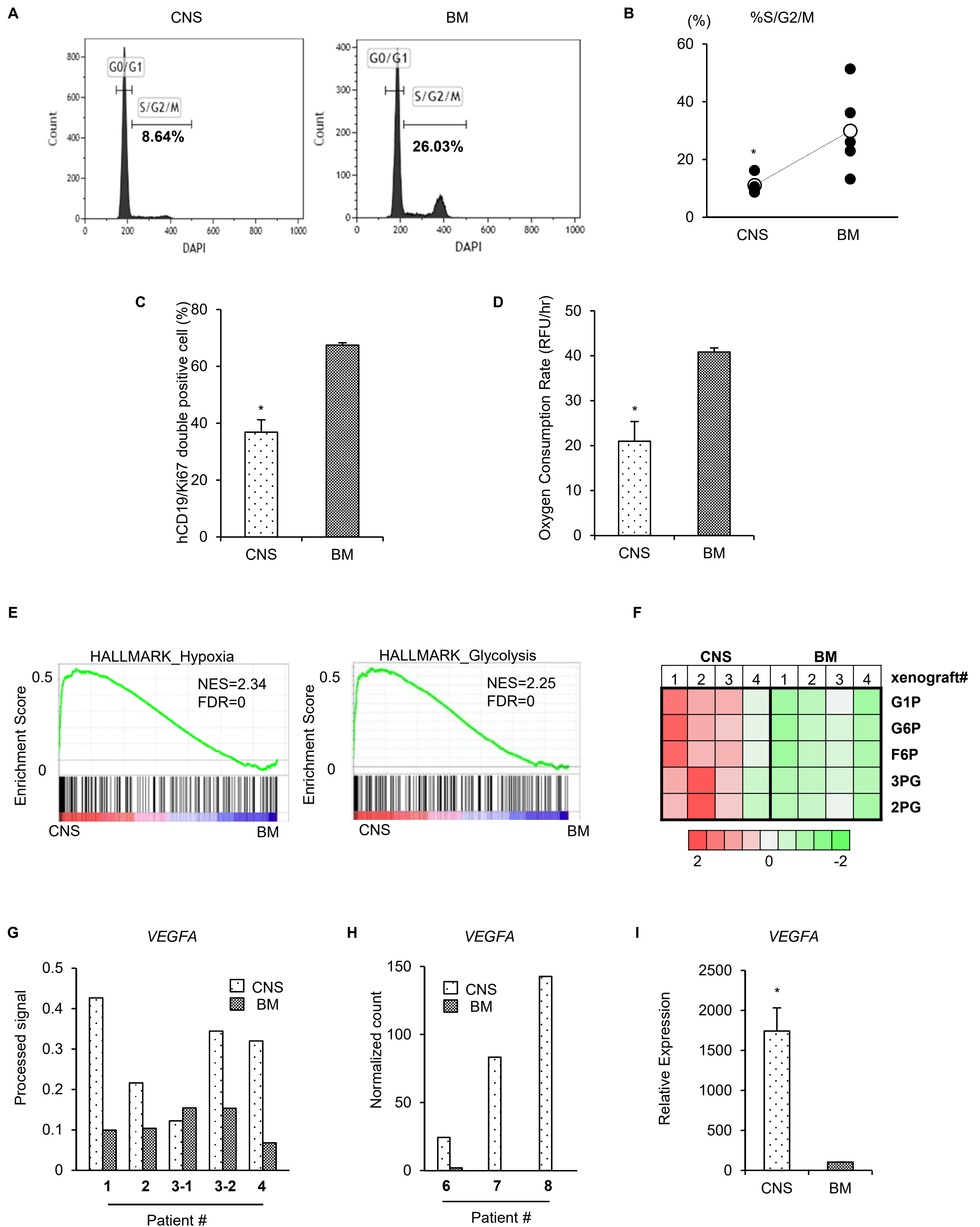

Figure 2