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J Neurooncol. Author manuscript; available in PMC 2014 August 15.

Published in final edited form as:
J Neurooncol. 2013 September ; 114(3): 275–279. doi:10.1007/s11060-013-1182-7.

Limited detection of IgH gene rearrangements in plasma of patients with primary central nervous system lymphoma

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Electronic supplementary material The online version of this article (doi:10.1007/s11060-013-1182-7) contains supplementary material, which is available to authorized users.

Conflict of interest K.W.K., N.P. and B.V. are co-founders of Inostics and Personal Genome Diagnostics (PGDx), own stock, and are members of their Scientific Advisory Boards. Inostics and PGDx have licensed several patent applications from Johns Hopkins, on which K.W.K., N.P. and B.V. are inventors.

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Abstract

Chemotherapy-based treatment of patients with primary central nervous system lymphoma can lead to durable remissions and potentially cure in a fraction of patients. Accurate assessment of residual disease is necessary to determine the duration and success of treatment that cannot be achieved by contrast-enhanced imaging due to limited sensitivity and specificity. A tumor-derived blood-based biomarker, if detectable and quantifiable, could serve as a more specific and reliable marker for these patients. The goal of this study was to assess whether lymphoma-specific IgH rearrangements can be detected in plasma of patients with PCNSL. PCNSL tissue was analyzed by capturing and sequencing the IgH genomic regions (IgCap) using next generation sequencing with the Illumina platform. Plasma of patients with detected IgH rearrangement was then analyzed for presence of the respective rearrangement using polymerase chain reaction. Tumor tissue and matched plasma of five treatment-naïve patients with biopsy-proven PCNSL (mean age of 65.6 years; range 62–68 years) were analyzed. All patients had measurable contrast-enhancing disease on MRI at time of plasma collection. IgH rearrangements were identified in 4 of 5 analyzed PCNSL tissue samples. The respective rearrangement could be detected in the plasma of 1 patient (25 %) but not in the others. IgH rearrangements can be detected in tumor tissue of patients with PCNSL using IgCap, however, they are absent or only present in minimal quantities in plasma, even in treatment-natïve patients with bulky disease. Alternative strategies to develop circulating biomarkers for PCNSL patients need to be explored.

Keywords

Primary central nervous system lymphoma; Biomarkers; Gene rearrangements; Circulating tumor DNA

Introduction

Primary central nervous system lymphomas (PCNSL) account for about 4 % of tumors of the central nervous system. The incidence of PCNSL, however has been rising in the immunocompetent population over the last few decades, particularly in the elderly population [1, 2].

High-dose methotrexate-based therapies are the standard of care for newly diagnosed PCNSL. Several different regimens achieve response rates of greater than 50 % and durable remissions in many patients [3]. PCNSL is now considered a potentially curable disease; however, most patients still recur after initial therapy and many eventually die of their disease.

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In order to choose the appropriate duration of treatment, it is important to determine when patients have achieved a complete response (CR), which is the prerequisite for a potential cure.

Response assessment in PCNSL has commonly been based on the same response criteria used for other primary brain tumors [4, 5], and patients are considered to have achieved a CR when all contrast-enhancing lesions have disappeared on MRI. However, PCNSL is known to diffusely involve non-contrast enhancing areas, CSF and the eyes, so contrast enhancement alone is not a reliable marker of a CR in these cancers. In addition, complete resolution of all contrast enhancing areas is not always achieved and residual contrast enhancement does not necessarily mean that there is residual disease [6]. As a result, investigators have started to focus on circulating tumor markers to assess disease status in patients with PCNSL [7]. To be clinically useful, such a marker needs to be exquisitely specific. A particularly attractive circulating biomarker is circulating tumor DNA (ctDNA), as such somatic alterations are unique to tumor cells.

The concept of mutated ctDNA as a specific and potentially sensitive circulating tumor marker has been demonstrated in several solid tumors [8–10]. Fusion genes are other genetic alterations that can function as circulating tumor markers in malignancies. Chronic myelogenous leukemia is an example of a disease in which fusion genes are routinely quantified as measure of disease burden and used for assessment of residual disease. Additionally, a recent study showed that IgH gene rearrangements could be detected in plasma of patients with non-Hodgkin's lymphoma, suggesting this as a potentially useful and highly specific blood-based tumor marker for lymphomas [11]. Based on these data, we set out to determine whether a similar IgH-based approach would be feasible in patients with PCNSL, as described below.

Methods

Sample collection

Adult patients with newly diagnosed, previously untreated, biopsy-proven PCNSL with measurable areas of contrast enhancement were asked to participate in this study. Pretreatment MRI scans were reviewed. Histologic and immunohistochemical slides of the cases included in the study were reviewed by a neuropathologist (FJR). Informed consent was obtained using a protocol that was approved by the Institutional Review Board at Johns Hopkins University. Formalin-fixed paraffin-embedded (FFPE) tissue samples were collected and used for analysis. Plasma was collected in EDTA-containing tubes, centrifuged twice immediately after collection and stored at −80 °C. Plasma collection and processing was done the same way as in other circulating tumor DNA studies performed by our laboratory [8]. Plasma samples were only thawed once for DNA extraction and immediately processed to optimize sample quality.

DNA purification

DNA was extracted from 10,000 μl of plasma with a QIAamp circulating nucleic acid kit following the manufacturer's instructions (QIAGEN; Valencia, CA, USA). DNA was

extracted from tumor tissues using a Qiagen AllPrep kit following the manufacturer's protocol. The amount of amplifiable DNA was quantified by a real time PCR based assay that amplifies human repeated sequences [12].

Tumor illumina library preparation

Approximately 1.5–3 μg oftTumor genomic DNA from formalin-fixed paraffin-embedded (FFPE) samples was used to prepare libraries following Illumina's (Illumina, San Diego, CA, USA) protocol.

IgCap capture

The targeted capture region included V-gene exons plus the first 36 bp of the downstream introns, six J-gene exons plus the first 36 bp of the upstream introns, and all the D-gene exons. Capture of the regions of interest was performed as described before [11, 13]. Captured DNA libraries were sequenced with the Illumina GAIIx Genome Analyzer, yielding 150 (2 × 75) base pairs from the final library fragments. Sequencing reads were analyzed and aligned to human genome hg18 with the Eland algorithm in CASAVA 1.6 software (Illumina). The sequence information was processed in silico to identify rearranged sequences as previously described [11].

Confirmation of IgH rearrangements in tumor and plasma samples

The full V-D-J or D-J joint region sequence and 40 bp from either side of the joint were used for primer design. The PCR mixture (50 μl) contained various amounts of 5 μl 10 × Platinum Taq buffer (Life Technologies), template DNA, 0.2 μM of forward-reverse primer mixture, 67 mM Tris (pH 8.8), 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM b-mercaptoethanol, 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM dTTP, 6 % (volyvol) DMSO, and 0.5 units of Invitrogen Taq polymerase. PCR was performed as follows: 96 °C for 4 min; 45 cycles of 96 °C for 10 s, 59 °C for 10 s, and 72 °C for 30 s. The primers used for PCR are listed in Supplement Table 1; some primers represented sequences present in the normal genome, while others represented sequences unique to particular patient's rearranged IgH genes. PCR products were gel purified with a QIAquick Gel Extraction Kit (QIAGEN; Valencia, CA, USA) and cloned with a TA clone kit (Promega, Madison, WI, USA) according to the manufacture's protocols. Plasmid DNA was evaluated by Sanger sequencing.

Results

Five patients with newly diagnosed, previously untreated PCNSL were studied. The patient demographics and representative MRI images are shown in Table 1 and Fig. 1. All patients had bulky, contrast enhancing, intraparenchymal brain lesions indicating disruption of the blood–brain barrier (BBB; Fig. 1).

DNA isolated from lymphoma tissue was used to construct libraries suited for massively parallel sequencing according to the IgCap protocol (see methods). Between 13,248,664 and 28,400,917 tags were generated per patient (Table 2). The sequence information obtained was then processed in silico to identify rearranged sequences at the IgH locus. IgH

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rearrangements were detected in the tumor tissue of four of the five patients and each rearrangement was validated by PCR with primers flanking the rearranged region (Table 3; Supplement Table 1).

Next, we analyzed DNA from plasma of the four patients with an identifiable rearranged IgH gene in their lymphomas. Even after extensive cycles of PCR (50), easily adequate to detect single copies of rearranged sequences [14] rearrangements could only be detected in one of the four plasma specimens analyzed (Fig. 2). The one rearrangement that was found in plasma (Patient 1) was confirmed by direct sequencing of the PCR product; the DNA sequence was identical with the one that was detected in the corresponding lymphoma tissue.

The detectability of IgH rearrangements in plasma did not correlate with presence or absence of contrast enhancement on MRI, as all 5 patients had visible contrast enhancement at time of plasma collection.

Discussion

This study shows that IgH rearrangements can be detected in tumor tissue of patients with PCNSL and that these rearrangements can occasionally be detected in the blood. However, it appears that the detectability of circulating rearrangements is limited, even in patients with bulky, contrast-enhancing disease prior to chemotherapy. The reason for the limited detectability of IgH rearrangements in plasma of these patients is unclear. The BBB in contrast enhancing regions of lymphoma is altered (as evidenced by presence of contrast enhancement on MRI), and one would assume that DNA can be shed into the bloodstream through gaps in the disrupted BBB, in particular in PCNSL that have not yet been treated with chemotherapy. Similar findings have been described previously by Jahnke et al. [15, 16] who analyzed 24 consecutive patients with PCNSL for presence of IgH rearrangements in blood and bone marrow using a three framework region (FR) assay. IgH rearrangements were detected in 4 of 24 patients with matched tumor and blood rearrangements were found in two patients, a similar frequency of detection to our observation [15].

Based on these combined findings, it appears that only limited quantities of tumor DNA find their way into the blood stream in patients with PCNSL and that circulating lymphoma DNA of rearranged IgH does not appear to be a useful marker of disease burden due to its low frequency of detection.

The question remains if this biomarker may be of higher yield when analyzing other compartments such as the patients' CSF. ctDNA may be present at higher concentrations in CSF; however, this assay would be more difficult to use clinically as it would require repeat lumbar punctures.

Supplementary Material

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Acknowledgments

This work was supported by the Robert H. Gross Memorial Fund, The Virginia and D.K. Ludwig Fund for Cancer Research and NIH grants CA96888, P01CA015396, and P30CA006973. M.H. was recipient of a Conquer Cancer Center Young Investigator Award by the American Society of Clinical Oncology. We thank Cherie Blair and Katharine Judge for sample acquisition and Dr. Stuart A. Grossman for critical review of this manuscript.

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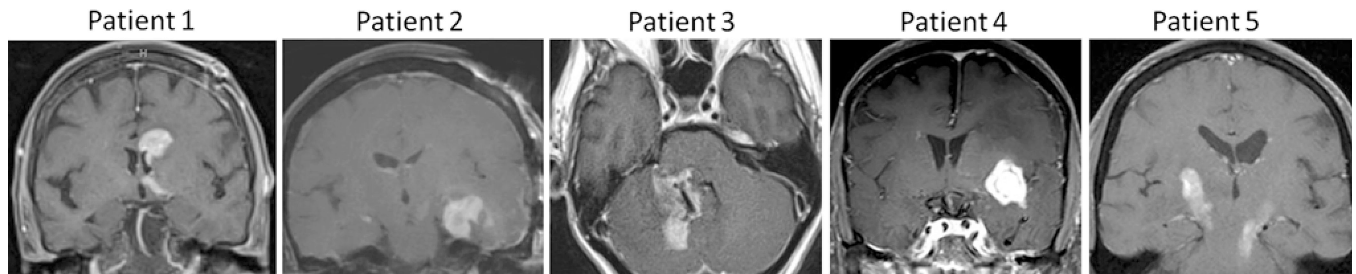

Fig. 1.

Contrast enhanced MRI images of patients prior to start of chemotherapy. Contrast enhancing areas have a disrupted BBB

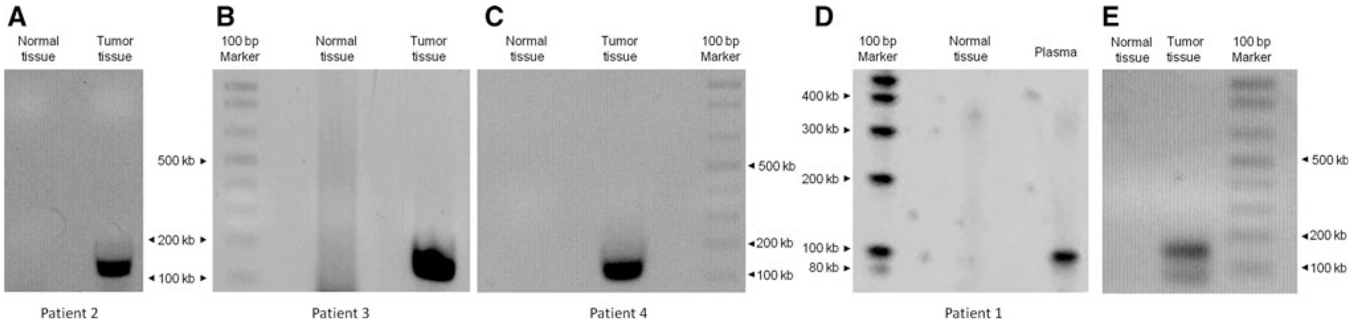

Fig. 2.

a–c Agarose gels showing amplified tissue DNA in patients #2–4; rearranged DNA was not detected in plasma of these patients (not shown). **d** Agarose gel showing amplified rearranged DNA in plasma after 50 cycles of PCR (patient #1); the DNA sequence of the band was sequenced and was found to be identical with the DNA of the lymphoma tissue. **e** Amplified rearranged tissue DNA of patient #1. Of note, the designed PCR product is 153 bp; the size is different from the rearranged DNA fragment in plasma (**d**) as different primers were used for tissue and plasma (positions of tissue and plasma primers that were used are illustrated in Supplement Fig. 1). Normal tissue DNA was used as negative control

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Table 1

Patient characteristics

Patient ID	Age at diagnosis (years), gender (M/F)	Location	Type of surgery	Diagnosis
Patient 1	67 F	Left frontal	Stereotactic biopsy	DLBCL
Patient 2	64 F	Left temporal	Partial resection	DLBCL
Patient 3	62 M	Cerebellar	Partial resection	DLBCL
Patient 4	67 F	Left frontotemporal	Stereotactic biopsy	DLBCL
Patient 5	68 M	Midbrain, pons	Stereotactic biopsy	DLBCL

DLBCL diffuse large B-cell lymphoma

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Table 2

Sequencing summary

Patient ID	Total tags	Tags matched uniquely to human genome	Tags matched to the Ig region	Tags matched to the Ig coding sequences	Average coverage of the Ig region	Target bases with more than 10 reads (number)	Target bases with more than 10 reads (%)[a]
Patient 1	13,248,664	5,726,682	577,050	360,693	1,018	16,029	90
	13,248,664	5,198,386	547,561	346,199	977	15,943	90
Patient 2	14,749,580.00	9,479,193	194,090	133,647	383	16,418	94
	14,749,580	9,254,038	192,561	132,646	380	16,339	94
Patient 3	28,400,917	16,497,095	746,102	475,937	1,362	16,029	92
	28,400,917	14,721,936	710,319	452,784	1,296	16,680	96
Patient 4	25,776,549	14,503,819	470,085	324,394	929	16,837	96
	25,776,549	12,604,082	444,729	306,449	878	16,828	96
Patient 5	13,545,846	5,079,690	372,873	251,786	711	12,679	71
	13,545,846	4,376,814	345,223	233,537	659	12,204	69

[a]Percent of bases with more than 10 reads of all bases that were attempted to be captured

Table 3

Rearrangements identified in this study

Patient ID	Rearranged sequence	Rearranged genes	Detected in plasma
Patient 1	AAAAACCAGTTCTCCCTGATGTTAGTTTCTGTGACTGCCATGGA CACGGGTGTCTATTTTTGTGCGAAGGCCCCGCGATTTGACTAC TGGGGCCAGGGAAACCTGGTCACCGTCGCCTCAGGTGAGTC ATCACAAC	IGHV4–61: J1	Yes
Patient 2	TGGACCCTCCTTGCTCCCTGGGAAGCTCCTCCTGACAGCCCC GCCTCCAGTTCCAGGTGTGGTTATTGTCAGGGGGTGTCAGACT GTGGTGGATACAGCTATGTGTACTGATATCTGGGGCCAAGGG ACAATGGTCAC	IGHD5–18: J3	No
Patient 3	TGAAGGGCCGATTCACCATCTCCAGAGACAACTCCAAGAACA CTCTCTCTCTGCAAATGACCAGCCTGAAAACTGAAGACACGG CTGTCTATTACTGTGCGGGCGATGTTATAGCAGCTCGTCAGAT AGGATACTTCGTATACTGGGGCCGGGGCACCCTGGTCACCGT CTCTTCAGGTGA	IGHV3–7: J1	No
Patient 4	GTTTCCCCAGGCCTGGCGGTAGGTTTGAAGTGAGGTCTGTGTC ACTGTGGTATTACTATGATAGTAGTGGTTGCAACTAAACATAC GCTATCGTGGCTGTTGCCCGTTGGATCACGGCCTGGTACTTCG ATCTCTGGGGCCGTGGCACCCTGGTCACTGTCTCCT	IGHD3–22: J1	No
Patient 5	ND	ND	N/A

ND not detected, *N/A* not applicable

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