Circulating cell-free DNA and IL-10 from cerebrospinal fluids aid primary vitreoretinal lymphoma diagnosis

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Primary vitreoretinal lymphoma (PVRL) is a rare variant of primary central nervous system lymphoma (PCNSL) that presents diagnostic challenges. Here, we focused on circulating cell-free DNA (cfDNA) and interleukin-10 (IL-10) isolated from cerebrospinal fluid. Twenty-three VRL patients (17 PVRL, 2 PCNSL/O, and 4 relapsed VRL, from 10/2018 to 12/2021) and 8 uveitis patients were included in this study. CSF samples from 19 vitreoretinal lymphoma patients had sufficient cfDNA for next-generation sequencing. Of these patients, 73.7% (14/19) had at least one meaningful non-Hodgkin lymphoma-related mutation. The characteristic MYD88L265P mutation was detected in the CSF of 12 VRL patients, with a sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 63.2%, 100%, 100%, and 46.2%, respectively. No meaningful lymphoma related mutations were found in CSF samples from uveitis controls with typical intraocular lesions. Meanwhile, CSF IL-10 levels were elevated in 95.7% of the VRL patients, with a sensitivity, specificity, PPV, and NPV of 95.7%, 100%, 100% and 88.9%, respectively. Key somatic mutations like MYD88L265P and CD79B detected from CSF cfDNA and elevated CSF IL-10 levels can be promising adjuncts for primary vitreoretinal lymphoma diagnosis.

KEYWORDS
cerebrospinal fluid, circulating cell-free DNA, IL-10, MYD88, vitreoretinal lymphoma
Introduction

Primary vitreoretinal lymphoma (PVRL) is a rare extranodal non-Hodgkin lymphoma of the retina, vitreous, and optic nerve. Most PVRL patients are of the B-cell lineage; approximately 80% develop intracranial progression eventually, while 15%–20% of patients with primary central nervous system lymphoma (PCNSL) have intracranial involvement at diagnosis (1–4). Thus, PVRL is also considered a subset of PCNSL. Early diagnosis of vitreoretinal lymphoma benefits survival. PVRL often masquerades as chronic posterior uveitis, sometimes as retinitis pigmentosa of the retina, vitreous, and optic nerve.

Meanwhile, elevated aqueous humor or vitreous fluid IL-10/IL-6 >1; (3) vitreous pathology showing neoplastic cells; (4) positive vitreous cell immunoglobulin gene rearrangement (IgH, Igκ, or Igλ); and (5) vitreous flow cytometry positive for lymphoma biomarkers. VRL patients with no evidence of CNS or systemic lymphoma were considered PVRL. Sometimes asymptomatic concurrent intracranial lesions were found in the routine head MRI; these VRL patients were considered PCNSL/O. PVRL patients were those who had previously been treated for systemic lymphoma (n=2) or vitreoretinal lymphoma (n=2), now experienced restricted intraocular relapse. Additionally, eight uveitis patients who presented with typical vitreous opacities and subretinal lesions were enrolled from 11/2019 to 12/2021 as suspected vitreoretinal lymphoma cases. Thorough exams ruled out the possibility of VRL; no malignancy presented on follow-ups (4.5–29 months).

This study conformed to the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Peking Union Medical College Hospital. Written informed consent was obtained from each participant. Furthermore, patients with VRL received lumbar puncture at baseline, before each chemotherapy and every 6 months during maintenance therapy to examine CSF and rule out CNS progression. Ten microliters of cerebrospinal fluid (CSF) and buccal mucosa were obtained from each patient prior to treatment for sequencing. Data on clinical characteristics were collected from electronic health records.

DNA extraction, library preparation, and targeted DNA sequencing

Germline DNA was extracted from buccal mucosa using the DNeasy Tissue Kit (Qiagen, USA) according to the
result of sequencing. As for validity measurement, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated (52).

Statistical methods

RStudio was used to present the results of sequencing. As for validity measurement, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated (52).

Result

CSF circulating cell-free DNA in patients with vitreoretinal lymphoma

We collected CSF samples from 23 patients with B-cell vitreoretinal lymphoma, with or without CNS involvement and lymphoma history (Table 1). Specifically, 17 patients with primary vitreoretinal lymphoma, 2 VRL patients with concomitant CNS lymphoma, 2 patient whose vitreoretinal lymphoma had relapsed, and 2 patients whose previous systemic DLBCL had intraocular relapse were included in this study. Additionally, eight patients who presented with typical vitreous and subretinal lesions but without evidence of malignancy were included as controls, including idiopathic uveitis, cytomegalovirus retinitis, ocular sarcoidosis, and radiation retinopathy. All patients with suspected vitreoretinal lymphoma underwent several diagnostic tests, as shown in the schematic flowchart (Figure 1).

cDNA was extracted from CSF; then, targeted deep sequencing of NHL-related genes was performed to identify somatic mutations (Figures 2, 3). In four patients with primary vitreoretinal lymphoma, the amounts of extracted cDNA (ranged from 0.1 to 0.6 ng) were not sufficient for cDNA library construction, which failed to perform sequencing. Analysis of the cDNA in the CSF of the remaining 19 vitreoretinal lymphoma patients revealed detectable mutations in 14 patients (Figure 2), at different variant allele frequencies (VAFs), ranging from 1.0% to 96.9%. In PVRL and PCNSL/O, 11/15 of the sequenced patients had MYD88L265P mutation, while 5 were with MYD88L265P and CD79B co-mutation. PIM1 was the most frequently mutated gene. In the meantime, sequencing of non-lymphoma controls’ CSF cDNA showed no mutation in five, insufficient cDNA in two, and DNMT3A c.1851+1G>A mutation (VAF 0.9%) in one (CONTROL-5).
CSF cfDNA is more sensitive than vitreous histology or flow cytometry in detecting vitreous–retina lesions at baseline

The detection of cfDNA in CSF was compared with the conventional methods of malignant cells identification at baseline (Table 2) and diagnostic validity was compared (Table 3). Although neoplastic lymphocytes were found in 22 out of the 23 VRL patients, most of the biopsy samples were not enough for immunohistochemical staining. Only four VRL patients were diagnosed with histology. Vitreous flow cytometry detected malignant B cells in 9 out of the 21 VRL patients tested, whereas vitreous cell immunoglobulin gene rearrangement detected 18 out of the 23. In our cohort, CSF cfDNA analysis revealed NHL-related gene mutations in 73.7% of the vitreoretinal lymphoma patients, with higher sensitivity than vitreous histology or flow cytometry (17.4% and 42.9%, respectively), slightly lower sensitivity than vitreous Ig gene rearrangement (78.3%). Notably, in the 14 cases that exhibited cfDNA mutation, 12 were with MYD88L265P mutation (overall sensitivity of 63.2%), and 6 were with MYD88L265P and CD79B co-mutation. Although in some cases lymphoma cells were not detected by cytology (PVRL-02, PVRL-05, PVRL-11, and

| ID     | Diagnosis  | Gender | Age | Intraocular lesions | Intraocular lesion location | Extraocular lesions at diagnosis | Previous disease |
|--------|------------|--------|-----|---------------------|-----------------------------|---------------------------------|------------------|
| PVRL-01| PVRL       | F      | 61  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |
| PVRL-02| PVRL       | M      | 48  | Left               | Vitreous + subretinal       | NA                              | NA               |
| PVRL-03| PVRL       | M      | 52  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |
| PVRL-04| PVRL       | F      | 48  | Bilateral          | Vitreous                    | NA                              | NA               |
| PVRL-05| PVRL       | F      | 70  | Bilateral          | Vitreous                    | NA                              | NA               |
| PVRL-06| PVRL       | F      | 52  | Right              | Vitreous + subretinal       | NA                              | NA               |
| PVRL-07| PVRL       | F      | 69  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |
| PVRL-08| PVRL       | F      | 44  | Bilateral          | Vitreous                    | NA                              | NA               |
| PVRL-09| PVRL       | F      | 54  | Bilateral          | Vitreous                    | NA                              | NA               |
| PVRL-10| PVRL       | M      | 62  | Right              | Vitreous + ciliary body     | NA                              | NA               |
| PVRL-11| PVRL       | F      | 56  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |
| PVRL-12| PVRL       | F      | 69  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |
| PVRL-13| PVRL       | M      | 69  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |
| PVRL-14| PVRL       | M      | 39  | Bilateral          | Vitreous                    | NA                              | NA               |
| PVRL-15| PVRL       | F      | 41  | Bilateral          | Vitreous                    | NA                              | NA               |
| PVRL-16| PVRL       | F      | 51  | Left               | Vitreous + subretinal       | NA                              | NA               |
| PVRL-17| PVRL       | F      | 49  | Left               | Vitreous + subretinal       | NA                              | NA               |
| PCNSL/O-21| PCNSL/O | M     | 61  | Bilateral       | Vitreous + subretinal       | Right frontal lobe          | NA               |
| PCNSL/O-22| PCNSL/O | M     | 62  | Bilateral       | Vitreous + subretinal       | Multiple intracranial lesions | NA               |
| RVRL-31| RVRL       | F      | 61  | Right              | Vitreous                    | NA                              | PVRL(Left)       |
| RVRL-32| RVRL       | F      | 50  | Bilateral          | Vitreous + subretinal       | NA                              | DLBCL (breast, bone) |
| RVRL-33| RVRL       | F      | 58  | Bilateral          | Vitreous                    | NA                              | DLBCL            |
| RVRL-34| RVRL       | F      | 52  | Right              | Vitreous + subretinal       | NA                              | PVRL (Bilateral) |
| CONTROL-1| Idiopathic uveitis | M  | 64  | Right              | Vitreous + subretinal       | NA                              | PCNSL            |
| CONTROL-2| Idiopathic uveitis | F  | 58  | Left               | Vitreous + subretinal       | NA                              | NA               |
| CONTROL-3| Idiopathic uveitis | F  | 57  | Right              | Vitreous + subretinal       | NA                              | NA               |
| CONTROL-4| CMV retinitis | F  | 62  | Bilateral          | Vitreous + subretinal       | NA                              | AITL             |
| CONTROL-5| Ocular sarcoidosis | M  | 57  | Bilateral          | Vitreous + subretinal+ciliary body | NA                              | NA               |
| CONTROL-6| Radiation retinopathy | F  | 47  | Right              | Vitreous + subretinal       | NA                              | PCNSL            |
| CONTROL-7| Idiopathic uveitis | F  | 58  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |
| CONTROL-8| Idiopathic uveitis | M  | 61  | Bilateral          | Vitreous + subretinal       | NA                              | NA               |

AITL, angioimmunoblastic T-cell lymphoma; CMV, cytomegalovirus; DLBCL, diffuse large B-cell lymphoma; NA, not applicable; PCNSL, primary central nervous system lymphoma; PCNSL/O, primary central nervous system lymphoma and intraocular involvement; PVRL, primary vitreoretinal lymphoma; RVRL, relapsed vitreoretinal lymphoma.
PCNSL/O-21&22), the characteristic MYD88<sup>L265P</sup> mutation was detected in the CSF cfDNA (Figure 2). These findings suggest that sequencing CSF cfDNA can act as an adjunct approach to the diagnosis of VRL.

**IL-10 levels are elevated in the CSF of vitreoretinal lymphoma patients**

CSF IL-10 was previously demonstrated as a biomarker for the diagnosis and prognosis of primary central nervous system large B-cell lymphoma, with a cutoff value of 8.2 pg/ml (24). Here, we found that the levels of CSF IL-10 were also elevated in 22 out of the 23 vitreoretinal B-cell lymphoma patients, while the CSF IL-10 levels were within normal limits in the control uveitis group. Elevated CSF IL-10 levels had a sensitivity, specificity, PPV, and NPV of 95.7%, 100%, 100%, and 88.9% for the diagnosis of VRL, whereas those were 95.7%, 62.5%, 81.5%, and 75% for vitreous IL-10 levels. Our findings provide evidence that CSF IL-10 could be a good diagnostic marker for primary vitreoretinal lymphoma.
FIGURE 3
Single and multiple somatic mutations of cerebrospinal cfDNA in vitreoretinal lymphoma patients at baseline.

TABLE 2 Diagnostic tests of vitreoretinal lymphoma patients and uveitis controls.

| ID    | Histology            | Vitreous cell Ig gene rearrangement | Vitreous FCM (pg/ml) | Vitreous IL-10/IL6 | CSF FCM | CSF cell number (×10⁶/L) | CSF WBC number (×10⁶/L) | CSF cfDNA MYD88L265P | CSF IL-10 (pg/ml) |
|-------|----------------------|------------------------------------|----------------------|--------------------|---------|--------------------------|------------------------|----------------------|------------------|
| PVRL-01 | Neoplastic lymphocytes | N                                   | P                    | 1,179.7            | N       | 74                        | 12                     | P                    | 472              |
| PVRL-02 | Neoplastic lymphocytes | P                                   | N                    | 150.7              | N       | 6                         | 2                      | P                    | 252              |
| PVRL-03 | Neoplastic lymphocytes | P                                   | P                    | 71.2               | N       | 12                        | 0                      | P                    | 136.0            |
| PVRL-04 | NC                   | P                                   | P                    | 2,588.3            | N       | 0                         | 0                      | N                    | 69.4             |
| PVRL-05 | Neoplastic lymphocytes | P                                   | N                    | 589.8              | N       | 2                         | 0                      | P                    | 35.7             |
| PVRL-06 | Neoplastic lymphocytes | P                                   | P                    | –                  | N       | 4                         | 0                      | P                    | 82.2             |
| PVRL-07 | Neoplastic lymphocytes | N                                   | P                    | 251.2              | N       | 4811                       | 10                     | P                    | 108.0            |
| PVRL-08 | Neoplastic lymphocytes | P                                   | P                    | 282.3              | N       | 6                         | 2                      | IS                   | 35.0             |
| PVRL-09 | DLBCL                | P                                   | ND                   | 862                | <1      | 6                         | 2                      | IS                   | 5.0              |
| PVRL-10 | Ciliary body–DLBCL   | N                                   | N                    | 1,562.3            | 2.4     | ND                        | 0                      | IS                   | 35.0             |
| PVRL-11 | Neoplastic lymphocytes | P                                   | N                    | >10,000            | >102     | N                         | 2                      | 2                    | P                |
| PVRL-12 | DLBCL                | N                                   | N                    | –                  | 12.5    | N                         | 14                     | 8                    | P                |
| PVRL-13 | Neoplastic lymphocytes | P                                   | P                    | 1,089.7            | 90.8    | N                         | 26                     | N                    | 83.0             |

(Continued)
### TABLE 3 Diagnostic validity of different diagnostic tests for vitreoretinal lymphoma.

|                | CSF MYD88 L265P | CSF IL-10 | Vitreous cytology | Vitreous flow cytometry | Vitreous Ig gene rearrangement | Vitreous IL-10 |
|----------------|----------------|-----------|-------------------|-------------------------|-------------------------------|---------------|
| **Sensitivity** | 63.2%          | 95.7%     | 17.4%             | 42.9%                   | 78.3%                         | 95.7%         |
| **Specificity** | 100%           | 100%      | 100%              | 100%                    | 100%                          | 100%          |
| **Positive predictive value** | 100%          | 100%      | 100%              | 100%                    | 100%                          | 100%          |
| **Negative predictive value** | 46.2%         | 88.9%     | 32%               | 36.8%                   | 58.3%                         | 75%           |

The presented histology results were from vitrectomy, if not specified.

DLBCL, diffuse large B-cell lymphoma; IS, insufficient cfDNA for NGS; N, negative; NC, no cells detected; ND, not done; P, positive.
Baseline CSF cfDNA levels or IL-10 level cannot predict treatment response

To determine whether baseline CSF biomarkers have a prognostic potential, we corrected cfDNA levels and their maximal somatic variant allelic frequency (maxVAF) with patients' clinical outcome–progression-free survival time in the five patients treated with R2 (Rituximab combined with lenalidomide), as shown in Table 4. CSF cfDNA amount and maxVAF did not correlate with VRL PFS (p= 0.89, 0.55, respectively). Neither was the CSF IL-10 level (p=0.47).

Discussion

The diagnosis of primary vitreoretinal lymphoma is still challenging. PVRLs usually present with bilateral blurry vision and floaters, anterior segment findings of keratic precipitates, and vitreous cellular infiltration of various severities (53). The clinical manifestations of primary vitreoretinal lymphoma are rather masquerading; patients are often misdiagnosed as intraocular inflammation or viral retinitis and wrongly treated (5–7). Cytological and immunohistochemical evidence of malignant lymphoma cells is the gold standard for diagnosis. However, the sensitivity of vitreous biopsy is disappointingly low, due to the lack of lymphoma cells in the vitreous specimen and necrosis during preparation (9). New diagnostic approaches like flow cytometry and molecular analysis of vitreous samples add to the diagnosis. Although Cani et al. (54) proposed with four patients that next-generation sequencing (NGS) test did not compromise the sample volume needed for other diagnostic tests, including cytology and flow cytology. From our experience, after cytology-based tests (cytology, immune cytology, and Ig rearrangement test) and flow cytology, the remaining vitreous samples could not provide enough DNA for NGS.

Hence, we wondered whether CSF could be a substitute marker for PVRL diagnosis, since PVRL is a special subset of PCNSL and previous studies have demonstrated the diagnostic role of CSF cfDNA and elevated IL-10 levels (24, 55). Furthermore, serial CSF monitoring might be promising in the early detection of CNS progression in vitreoretinal lymphoma patients. To address the unmet needs of PVRL diagnosis, we conducted this study to analysis the diagnostic roles of CSF biomarkers, circulating cell-free DNA, and IL-10.

**MYD88** L265P is a unique non-synonymous point mutation in B-cell malignancies (56). Several studies demonstrated the presence of **MYD88** L265P mutation in the aqueous humor and vitreous fluid of vitreoretinal lymphoma patients. In different PVRL cohorts, the reported sensitivity of **MYD88** L265P mutation detection ranged from 25% to 88.9%, with direct Sanger sequencing of polymerase chain reaction (PCR), droplet digital PCR, or sequencing (12–19). The vitreous fluid samples showed higher positive rate than paired aqueous humor samples (14).

We wondered whether **MYD88** L265P mutation also presented in the CSF of PVRL patients. In this study, we collected CSF samples from 31 patients with suspected VRL, then performed NGS. The final diagnosis was VRL in 23 patients. Despite the four samples without sufficient cfDNA for NGS, **MYD88** L265P mutation was confirmed in 12 of the remaining 19 VRL patients. Six patients were with **MYD88** L265P and **CD79B** co-mutation. Meanwhile, none of the uveitis controls contained the characteristic lymphoma mutations. The sensitivity, specificity, PPV, and NPV for using CSF **MYD88** L265P as VRL diagnostic marker were 63.2%, 100%, 100%, and 46.2%, respectively. Our findings suggest that key somatic mutations (i.e., **MYD88** L265P) detected from CSF samples can be a promising additional approach for the accurate diagnosis of VRL. Notably, mutations without specific clinical meanings might be detected in non-lymphoma patients, like low frequency **DNMT3A** splicing mutation.

With CSF samples, we were able to overcome the difficulty of insufficient vitreous biopsy samples and picture the genomic features of vitreoretinal lymphomas. This can be a promising adjunct to vitreous fluid samples genomic analyses (19). Although there have been no standard treatment protocols for vitreoretinal lymphomas, the baseline mutation information presents targets for potential precision therapy,

### Table 4 Baseline cfDNA and PVRL prognosis.

| ID   | Diagnosis | Treatment | Clinical outcomes | PFS (months) | Amount of cfDNA (ng) | maxVAF |
|------|-----------|-----------|-------------------|--------------|----------------------|--------|
| PVRL-01 | PVRL | R2 | (contralateral eye) | 20.9 | 5.04 | 86.8% |
| PVRL-02 | PVRL | R2 | (Bilateral precentral gyrus) | 9.2 | 2.10 | 94.8% |
| PVRL-03 | PVRL | R2 | | 25.0 | 0.37 | 61.8% |
| PVRL-04 | PVRL | R2 | | 11.0 | 0.60 | 61.8% |
| PVRL-05 | PVRL | R2 | (bilateral corpus callosum, cerebellum, lateral ventricle) | 8.2 | 2.32 | 25.5% |
which might improve prognosis. Furthermore, we also need disease monitoring biomarkers for PVRL patients. For early detection of disease relapse and CNS progression, biomarkers like MYD88\textsuperscript{L265P} or CD79B variant allele frequencies are promising. This study confirmed the presence of PVRL characteristic mutations in CSF, also established the foundation of assessing CSF samples to monitor disease progression. Furthermore, routine lumbar puncture for cerebrospinal fluid reduces the possible intraocular complications from aqueous puncture or vitreous aspiration.

IL-6 and IL-10 are the most extensively studied cytokines in vitreoretinal lymphomas; studies have demonstrated that elevated aqueous humor or vitreous fluid IL-10 and IL-10/IL-6 ratio can help distinguish vitreoretinal lymphomas from uveitis. However, there are also lymphoma cases with low IL-10 levels or non-lymphoma cases with elevated IL-10 levels (22, 57–59). In this study, we demonstrated that in patients with restricted intraocular lesions, 95.7% had elevated CSF IL-10 levels (ULN, 8.2 pg/ml), while the CSF IL-10 levels were within normal range in the controls. The sensitivity, specificity, PPV, and NPV were 95.7%, 100%, 100%, and 88.9%, respectively. The diagnostic accuracy of CSF IL-10 was slightly higher than vitreous fluid IL-10 (96.8% versus 80.6%). Meanwhile, parallel test of CSF MYD88\textsuperscript{L265P} and CSF IL-10 levels showed a sensitivity of 98.4% and specificity of 100%. Furthermore, we have been detecting the IL-10 levels in serial CSF samples after treatment to assess whether IL-10 can be a potential disease monitoring biomarker.

A significant limit of our study is the small cohort size. Future studies with larger cohorts are needed. Furthermore, cfDNA extraction procedure still needs optimization to eliminate the effect of cfDNA degradation and increase the quantity of extracted cfDNA for sequencing. Nevertheless, we demonstrate that meaningful molecular data can be obtained from CSF cfDNA in PVRL patients. CSF MYD88\textsuperscript{L265P} mutation and CSF IL-10 can be complementary approaches to the current diagnostic standard of PVRL. NGS of the CSF cfDNA also provides targets for precision therapy, including MYD88, CD79B, and CDKN2A. In the meantime, we have been collecting CSF samples of PVRL patients during therapy to investigate whether the mentioned biomarkers can monitor treatment response or indicate disease progression.

Conclusions

Our study provides mutation landscape of vitreoretinal lymphomas with next-generation sequencing. MYD88\textsuperscript{L265P} or CD79B mutation detected from CSF circulating cell-free DNA aids in primary vitreoretinal lymphoma diagnosis. Patientspecific genomic alterations are also pictured, which provide therapeutic targets for personalized medicine. Furthermore, IL-10 levels are also elevated in the CSF of VRL patients, with higher specificity than vitreous fluid IL-10.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: Link - https://ngdc.cnch.ac.cn/gsa-human/browse; Accession - HRA002732/HRA002732.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of Peking Union Medical College Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZZ, YZ, XZ, MFZ, and WZ designed the experiment. YZ, XZ, MFZ, and WZ enrolled participants and treated the enrolled patients. ZZ and DMZ collected patient samples and data. ZZ, DMZ, and LZ conducted the experiments. CWJ double checked all the histology samples. ZZ wrote the first draft manuscript. All authors edited and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.955080/full#supplementary-material

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