

Chemofailure and chemoresistance in retinoblastoma

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Retinoblastoma (RB) is the most common malignant intraocular tumor in children constituting 4% of all pediatric cancers.⁽¹⁾ RB1 gene mutation affects the developing retina resulting in this malignant tumor which is mostly diagnosed in infancy or in early childhood. The current management protocol employs chemotherapy as the primary modality for management of retinoblastoma.⁽²⁾ Tumor response to chemotherapy has increased the chances of cure manifold and the overall 3-year survival rate has increased to more than 90%.⁽³⁾ Chemotherapeutic drugs induce tumor regression and reduce tumor volume, thereby allowing effective control using focal therapies.⁽⁴⁾ The multimodal management of RB involving chemotherapy and focal treatment aim to preserve globe with functional vision⁽⁵⁾ and avoid the morbidities associated with external beam radiation⁽⁶⁾ and enucleation.⁽⁷⁾

Failure of Chemo-response

Despite the good clinical response of the tumor to multi-drug chemotherapeutic regimens, non-response of some tumors to chemotherapy and recurrence after chemotherapy continue to raise concern. Various authors have reported their experiences with retinoblastoma tumors that have failed to respond to chemotherapy. Chemotherapy response is defined as a decrease in tumor size, or displaying regression patterns⁽⁸⁾ with no progression to subretinal or vitreous seeding.⁽⁹⁾

Bartuma et al⁽¹⁰⁾ documented their 10-year retrospective experience of systemic chemotherapy in 46 eyes with hereditary retinoblastoma. Hereditary retinoblastoma was defined as any patient with bilateral tumors, unilateral familial or unilateral multifocal. Seven eyes were non-responsive to systemic chemotherapy with VEC (Vincristine/ Etoposide/ Carboplatin) and belonged to group B(n=1), group C(n=2), group D(n=3) and group E(n=2) International Classification of Retinoblastoma. Of these, one group D tumor and the 2 group E tumors required enucleation. The remaining 4 either received additional cycles of VEC or were switched to IVAd. The IVAd protocol induced tumor regression in all the tumors with VEC resistance. However, the favorable response in the two Group D tumors was temporary and they were enucleated. Enucleation, when done, was indicated for multiple subretinal and/or vitreous seeds and non-

responsive large tumor. Upon histopathological evaluation, 3 of the enucleated eyes displayed high-risk features including choroidal infiltration (n = 3) and anterior chamber invasion (n = 2). One patient developed osteosarcoma with lung metastases four years after diagnosis of retinoblastoma. This highlights the lifelong increased cancer risk of germline mutation carriers and substantiates the need for lifelong follow-up in these patients.

Intra-arterial chemotherapy(IAC) has been documented as a highly successful treatment option by various groups in tumor control in advanced retinoblastomas. It has also led to decreased systemic effects of intravenous chemotherapy such as myelosuppression, immunosuppression and the risk of secondary leukemia. Shields et al⁽¹¹⁾ reported globe salvage rates of 100% of group C, 100% of group D and 33% of group E tumors when IAC was used as primary treatment. Gobin et al⁽¹²⁾ have demonstrated globe salvage rates as high as 82% with IAC in primary cases with advanced intraocular tumors and 58% with secondary IAC. When IAC was used as secondary treatment by Muen et al⁽¹³⁾ for tumors that failed to regress with primary systemic IVC and/or local therapies, 80% control was achieved. Despite the high success rates, chemo-resistance, resulting in enucleation, continues to be a concern even with this tumor-targeted intra-arterial administration. The indications for enucleation of post-IAC eyes are a combination of factors such as primary or secondary IAC treatment, non-response, relapse, vitreous seeding, IAC complications.

In 2010, Vajzovic et al⁽¹⁴⁾ reported the persistence of viable tumor in 3 eyes with Group D RB treated with supraselective IAC melphalan as secondary treatment. On HP examination after enucleation, two of the three eyes had optic nerve invasion by viable tumor; 1 eye had non-massive choroidal invasion.

In a histopathological study of 8 eyes, enucleated after primary IAC for RB, by Eagle et al,⁽¹⁵⁾ enucleation was indicated in four eyes due to the presence of viable tumor. Two of them were recurrent which manifested as vitreous seeds. The other two were instances of poor chemoresponse of the tumor - one displayed no evidence of treatment response: it was also found to be well differentiated and contained numerous rosettes/areas of photoreceptor differentiation. The other contained a large, extensively necrotic Rb with

small foci of residual viable tumor consistent with partial treatment response.

Pavlidou et al⁽¹⁶⁾ reported a retrospective case series of 12 enucleated eyes with retinoblastoma that were resistant to intra-arterial chemotherapy with melphalan. 9% received primary intra-arterial melphalan and 91% received it as secondary therapy following failure of primary treatment with systemic chemotherapy. Histopathologic analyses of the enucleated eyes revealed viable tumor in 8 eyes and anterior segment seeding in 8 of the 12 eyes (67%). Anterior segment infiltration involved the ciliary body and/or ciliary muscle (7 eyes [58%]), iris (6 eyes [50%]), and cornea (4 eyes [33%]). Among the 8 eyes with viable tumor, 6(50%) were poorly differentiated tumor and 2 (17%) were moderately differentiated. The causes for IAC failure could be attributed to the higher prevalence(55%) of germline tumors which are more aggressive. Furthermore, these eyes received monotherapy with melphalan. Addition of intra-arterial topotecan could have led to better tumor control. The anterior segment seeding could be due to the partial tumor necrosis induced by chemotherapy, thereby resulting in dispersion of seeds and their subsequent anterior movement.

Persistence of vitreous seeds is the main reason for globe loss after IAC. Intraophthalmic artery chemotherapy offered control for 82% of the cases without subretinal seeds but only 64% to 67% for those with vitreous seeds.⁽¹⁷⁾ The persistence of vitreous seeds after IAC may be explained by the presence of blood-retinal barrier and lack of vitreous blood flow preventing therapeutic IAC concentrations in the vitreous, and also drug resistance.⁽¹⁸⁾ Recently, intravitreal chemotherapy has become the preferred protocol for management of vitreous seeds.⁽¹⁹⁾

Chemoresistance

Failure of chemotherapy to induce tumor regression in advanced retinoblastoma has prompted evaluation of the mechanisms of drug resistance in RB. Drug resistance is a major factor that limits the effectiveness of chemotherapy.⁽²⁰⁾ Tumor cells may be intrinsically resistant prior to chemotherapy, or resistance may be acquired by tumors, during treatment, that were initially sensitive to chemotherapy.

Meeteren et al⁽²¹⁾ studied the histopathological features in 44 enucleated eyes of primary retinoblastomas unexposed to chemotherapy nor radiotherapy and correlated to in vitro drug resistance. Undifferentiated retinoblastoma was sensitive to carboplatin, doxorubicin, ifosfamide, and thiotepa. Calcified tumors were more sensitive to vincristine. Vincristine inhibits mitosis by disrupting spindle formation, thus causing metaphasic arrest. Tumors with a high number of apoptotic cells are more resistant to vincristine and more sensitive to ifosfamide.

Causative factors of chemo-resistance-

Chemoresistance may be due to various factors affecting drug sensitivity such as accelerated drug efflux, DNA methylation, evasion of apoptosis, alterations in drug target and processing of drug-induced damage.⁽²⁰⁾

Accelerated drug efflux

Increase in drug efflux is often responsible for enhanced drug resistance and is frequently due to increased expression of ATP binding cassette (ABC) transporter proteins.⁽²²⁾ Many cytotoxic drugs such as vinca alkaloids, etoposide, anthracyclines(doxorubicin) taxanes and mitomycin C, topotecan are targets for the ABC transporter proteins. Chemo-resistance has most often been linked to overexpression of one such protein - Permeability glycoprotein or P-glycoprotein (Pgp). P-gp confers resistance by mediating the ATP-dependent efflux of a wide array of anticancer drugs with differing mechanisms of action resulting in the multidrug-resistant phenotype in cancer.⁽²³⁾ In RB, the expression of P-gp has been related to the failure of chemotherapy.⁽²⁴⁾ In a study by Krishnakumar et al⁽²⁵⁾ on 60 eyes enucleated prior to chemotherapy, P-gp was expressed in 38% (23/60) of tumours. This shows that retinoblastomas express P-gp is intrinsically even prior to chemotherapy suggesting the existence of intrinsically resistant tumour cell clones in retinoblastoma that could possible contribute to instances of failure of primary chemotherapy.

The degree of tumour differentiation as well as the expression of P-gp have been linked to the chemoresistance of retinoblastoma.⁽²⁶⁾ Filho et al⁽²⁷⁾ analysed and correlated P-gp expression with histopathological features of RB treated with chemotherapy prior to enucleation. The histopathological features studied included the degree of differentiation as well as optic nerve and choroidal invasion. Of the 17 enucleated eyes, nine were treated with only chemotherapy; 8 received chemo along with focal therapy. Viable tumor cells were present in all cases that received only chemotherapy. P-gp expression was noted in 16 (94%) eyes. P-gp positivity was observed in tumor areas containing rosettes and viable tumour cells. In eyes with high-risk features like optic nerve and massive choroida invasion, P-gp was expressed in the infiltrating retinoblastoma cells. Of the nine RB treated with chemotherapy alone, six (66.6%) were regressed with well-differentiated cellular component and three (33.4%) had viable cells with poor differentiation. The degree of cellular differentiation of RB cells can affect the clinical response to chemoreduction similar to its influence on the response to irradiation, with highly differentiated components of RB being relatively resistant to these treatments. Tumour recurrences of RB after chemotherapy suggest inherent insensitivity of RB to chemotherapy.

P-gp could be inherently over-expressed in cancer cells or may be acquired, a feature generated by exposure to anticancer drugs. Kashyap et al⁽²⁸⁾ compared the expression of P-gp in RB eyes after primary enucleation and after secondary enucleation following systemic chemo. 27% Vs. 58.3% This high expression of P-gp post-chemo may point towards the selection of these resistant clones of P-gp-expressing cells.

Inhibiting Pgp has been sought as a strategy to reverse multi-drug resistance.⁽²³⁾ Chan's group advocated the addition of cyclosporine A, which blocks P-gp, to chemotherapeutic regimen in order to control intraocular retinoblastoma and avoid radiation.⁽²⁹⁾ They reported a high cure rate of 92% in retinoblastoma patients who received primary treatment with cyclosporin A in combination with chemotherapy. However, the systemic toxicity was unacceptable for it to be incorporated into chemotherapy protocols.

Hypermethylation

Recent research suggests that many tumor suppressor genes such as RB1 are methylated and thus inactivated, leading to tumorigenesis.⁽³⁰⁾ DNA methylation represents one of the earliest identified epigenetic modification pathways. DNA methylation occurs by the addition of a methyl group at a 5' carbon group, usually at cytosine-guanosine dinucleotides (CpGs), resulting in gene silencing and inhibition of transcription. During progression of retinoblastoma, RB1 gene inactivation is followed by additional genomic modifications which progressively lead to resistance of tumor cells to death.⁽³¹⁾

Greger et al⁽³²⁾ in 1989 reported five unilateral RB patients with no mutation in the *RB1* gene. Instead, hypermethylation of the 5' end of the *RB1* gene, including its promoter region and exon 1 was observed in these patients. Promoter hypermethylation, and thus epigenetic silencing, of the *MGMT* gene (O6-methylguanine-DNA methyltransferase) was observed in 58% of retinoblastomas in a study that compared the methylation status in 12 RB tumors and their corresponding normal retinas.⁽³³⁾ *MGMT* hypermethylation was also associated with advanced stages of retinoblastoma.⁽³⁴⁾ Hegi et al⁽³⁵⁾ demonstrated hypermethylation of the *MGMT* promoter in 68% of glioblastomas. This was also observed to be a favourable prognostic marker with a longer overall survival when treated with the alkylating agent temozolomide paving the way for personalized precision medicine.⁽³⁶⁾

Poulaki et al⁽³⁷⁾ found that Fas-expressing Rb cells are resistant to death receptor (DR)-mediated apoptosis which was attributed to hypermethylation-mediated gene silencing of pro-apoptotic CASP8 gene. Hypermethylation of caspase-8 gene results in decreased caspase-8 protein expression.⁽³⁸⁾ The demethylating agent 5-aza-2'-deoxycytidine restored

caspase-8 expression and thereupon, sensitivity to DR-mediated apoptosis.⁽³⁷⁾ These findings herald the emerging era of epigenetic therapeutics. Demethylating agents may be used to reverse the epigenetic tilt and thereby redeem the effectiveness of chemotherapy. 5-Aza-2'-deoxycytidine is being used in the management of myelodysplastic syndrome (MDS)⁽³⁹⁾ and acute myeloid leukemia (AML).⁽⁴⁰⁾

Genome instability in retinoblastoma might arise through a mechanism that involves somatic instability and promoter methylation of DNA repair genes.⁽⁴¹⁾ The mismatch repair (MMR) system is critical for the maintenance of genomic stability. It scans newly synthesized DNA for errors arising during DNA replication and repairs them. *hMLH-1* gene is involved in mismatch repair (MMR) of DNA and is hypermethylated in RB.⁽⁴²⁾ Cisplatin resistance has been attributed to defects in the MMR system arising from hypermethylation of the *hMLH1* promoter.⁽⁴³⁾

Evasion of Apoptosis

Apoptosis plays a crucial role in the mechanism of action of many anti-cancer drugs. Circumventing apoptotic mechanisms could provide the evolutionary survival advantage to tumor cells during their malignant transformation, cancerous growth and evasion from being targeted by chemotherapeutic agents.

Cisplatin, a widely used in the chemotherapy regimen for the treatment of retinoblastoma, acts by forming DNA adducts which impair proper DNA replication and activate apoptotic pathways. While some tumors exhibit intrinsic resistance to cisplatin, a significant fraction of initially sensitive cancers eventually develop chemo-resistance. Downregulation of Bax, overexpression of anti-apoptotic bcl-2 and enhanced activity of PI3-K/Akt are some of the known mechanisms through which inhibition of apoptotic signals occurs in cisplatin-resistant tumor cells.⁽⁴⁴⁾

The Bcl-2 gene has been shown to be overexpressed in many solid tumours cell lines, contributing to resistance to chemotherapy and radiotherapy.⁽⁴⁵⁾ Clinically, several studies have shown that high Bcl-2 expression correlates with a poor response to chemotherapy.⁽⁴⁶⁾ Loss of Bax expression results in increased resistance to chemotherapy.⁽⁴⁷⁾ Pillet et al⁽⁴⁸⁾ analyzed the pro-death effect of Bcl-2/Bcl ABT-737 on two human retinoblastoma cell lines, Y79 and WERI-Rb and found that the WERI-Rb cells were sensitive to ABT-737. However Y79 cells were resistant, probably due to the absence of Bax.

Survivin belongs to the family of cellular inhibitors of apoptosis proteins (cIAPs) that inhibit apoptosis by binding to active caspases, such as caspase-3, -7 and -9. Clinically, low levels of surviving in retinoblastoma have been correlated with a better response to chemotherapy.⁽⁴⁹⁾

Clusterin is a protective chaperone protein that protects various retinal cells. It is overexpressed in

many malignant tumors whose chemoresistance correlated with the expression of clusterin.⁽⁵⁰⁾ Song et al⁽⁵¹⁾ found that clusterin was highly expressed in human retinoblastoma tissues and cell lines (SNUOT-Rb1 and Y79). Transfection of SNUOT-Rb1 cells to increase clusterin expression resulted in inhibition of cisplatin-induced apoptotic cell death. This was confirmed by attenuated levels of cleaved caspase-3.

Role of basic-Fibroblast Growth Factor (bFGF)

bFGF plays contrasting roles in tumor cell biology. Studies show that bFGF induces chemoresistance in malignancies like small cell lung cancer, while sensitising others such as primitive neuroectodermal tumors and Ewing's sarcoma.⁽⁵²⁾

Cebulla et al⁽⁵³⁾ studied the role of bFGF in the pathobiology of RB. They established that bFGF is expressed in human RB cell lines, primary tumors, and transgenic murine RB. bFGF induced proliferation in two RB cell lines, and resulted in chemoresistance to carboplatin-induced apoptosis in the aggressive Y79 line. The Y79 line had a higher ratio of the high-molecular weight isoform to low molecular weight forms, compared with the more indolent WERI line, explaining the former's chemoresistance. In transgenic murine RB, bFGF was found to be upregulated during tumorigenesis, with the peak coinciding with early tumor formation (8 weeks). This finding supports the existence of an "angiogenic switch" in which a proangiogenic pattern of gene expression occurs, preceding tumor spread.

Role of L1CAM

L1CAM is an adhesion molecule that is involved in proliferation, migration, invasion, metastasis, and chemoresistance of cancer cells.⁽⁵⁴⁾ Jo et al⁽⁵⁵⁾ demonstrated that LCAM1 positivity was inversely related to the degree of tumor differentiation in retinoblastoma tumors and directly related to chemoresistance to carboplatin in RB cell lines.

Stemness as the probable reason for chemoresistance

The ATP-binding cassette sub-family G member 2 (ABCG2) is a multi-drug resistance transporter and also a neural stem cell marker.⁽⁵⁶⁾ Its expression in tumor cells is correlated with their resistance to chemotherapeutic agents and decreased survival rates.⁽⁵⁷⁾ A number of studies have established expression of stem cell/ chemoresistance marker ABCG2 in RB tumor cells.^(58,59,60) Approximately, 4% of retinoblastoma cells express ABCG2, with co-localization with other stem cell markers, such as Oct3/4 and Nanog.⁽⁵⁸⁾

Cassidy et al⁽⁶¹⁾ compared ABCG2+ and ABCG2-enriched Rb cell populations in three dimensional cultures and showed that ABCG2+ aggregates exhibited greater immunoreactivity to stem cell markers

ALDH1A1 and CD164, but less immunoreactivity to mature markers (MAP-2 and S-Antigen) as compared with ABCG2- cells. CD164 is a cell adhesion molecule found on the surface of stem cells and plays a role in the growth and metastasis in colon cancer cells. ALDH1A1 is another marker common to both developmental stem cells and cancer stem cells. These studies point towards the possibility that the resistance to chemotherapeutic agents may be due to the presence of clones of stem cells - cancer stem cells(CSCs).

Putative role of Cancer stem cells(CSCs)

The presence of CSCs was first demonstrated in acute myeloid leukemia(AML). Cancer stem cells are endowed with properties of immortality similar to other stem cells. They are slowly dividing, chemoresistant, and are also suspected to play a role in sustaining tumor progression and inducing relapse.⁽⁶²⁾ CSC sub-population have been identified in retinoblastoma as well.⁽⁶³⁾ The origin of these cancer stem cells could be:

- a. Clones of stem cells inherently present in the tumor giving credence to the "CSC as cell-of-origin" hypothesis⁽⁶⁴⁾
- b. Cellular re-programming: De-differentiation of tumor cells to assume stem cell-like attributes.^(65,66) This may be cause for the acquired resistance after chemotherapy.

Future prospects

Cellular pliancy is the property of cells to confer susceptibility to either death or cell-cycle re-entry. The mechanism underlying cellular pliancy is the organization of the epigenome. Cells with high pliancy adapt themselves to become more resistant to death by apoptosis or necrosis, but this state may also confer susceptibility to acquiring malignant properties because they can survive long enough to acquire all the hallmarks of cancer.⁽⁶⁷⁾ Epigenetic changes are reversible unlike genetic mutations. With rapidly advancing technological capabilities in epigenetic studies, it is envisioned that these molecular intricacies with translational significance would be unraveled. The altered epigenetic landscape, conferring chemoresistance to tumor cells, need to be identified and reversed in to using epigenetic drugs thereby resulting in huge strides in precision medicine for retinoblastoma management.

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