Evidence of Tumour Microenvironment and Stromal Cellular Components in Retinoblastoma

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Keywords

Retinoblastoma · Stromal cells · Tumour microenvironment · Endothelial cells · Fibroblasts · Tumour-associated macrophages

Abstract

Background: The tumour microenvironment (TME) consisting of tumour cells and multiple stromal cell types regulate tumour growth, invasion and metastasis. While the concept of TME and presence of stromal cellular components is widely established in cancers, its significance in the paediatric intraocular malignancy, retinoblastoma (RB), remains unknown. **Methods:** The study qualitatively identified the presence of multiple stromal cellular subtypes in RB TME by immunohistochemistry. **Results:** Results of the study identified the presence of stromal cell types such as endothelial cells, tumour-associated macrophages, fibroblasts, cancer-associated fibroblasts, retinal astrocytes and glia in RB TME. The extent of stromal marker positivity, however, did not correlate with histopathological features of RB. **Conclusions:** The findings of the study convincingly suggest the presence

of a stromal component in RB tumours. The interactions between stromal cells and tumour cells might be of profound importance in RB progression.

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Introduction

Retinoblastoma (RB) caused by the mutation in the RB1 gene [1, 2] and amplification of N-Myc gene [3] has an incidence of about 9,000 cases every year worldwide [4]. Primary therapeutic modalities used for RB majorly include chemotherapy via systemic, intra-arterial, intra-vitreous, periocular routes and are supported by focal therapy [5–9]. Limitations associated with chemotherapy include the development of drug resistance and other secondary complications [5, 10, 11].

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The complex mechanisms of chemoresistance observed in tumour cells are now being attributed to the interactions occurring in the tumour microenvironment (TME) [12]. Components of TME comprising tumour cells, immune cells and other stromal cells along with their vital interactions contribute to treatment response and disease prognosis. Accumulating evidence in both epithelial [13–16] and neural cancers [17, 18] suggest a close interplay between the tumour and niche involving non-neoplastic components to secrete signalling molecules, growth factors that contribute to increased angiogenesis, invasion, progression and metastasis [19–23]. The TME components are hence referred to as “partners in crime” for the tumourigenesis process [24, 25].

In addition to providing mechanistic insight into tumourigenesis, the knowledge on stromal components may also have direct clinical implications, since the role of the stroma is context-specific. For instance, in pancreatic cancers, several targeted therapies have failed during the clinical trials due to the presence of a highly heterogeneous and thick stromal microenvironment. It is suggested that, while on the one hand the stromal microenvironment restrains the tumour and prevents the metastasis, on the other hand, it also prevents most drugs from penetrating [26]. Therapies targeting stromal components are being considered, and this does not compromise the restraining part of the stroma while allowing the drugs to penetrate efficiently. The stromal cell populations in the tumour were studied using a long-term spinning disk confocal microscopy technique. Results showed stromal cells population to be more dynamic and evolving as the tumourigenesis progressed. This directly relates to the effect of therapies during stages of the tumour development. In addition to the tumour cells, the evaluation of the stromal microenvironment during pre-treatment, treatment and post-treatment may hence be required to decide on an ideal therapeutic regimen [27]. Emerging live imaging techniques such as diffusion-weighted imaging, which measures the mobility of water in the tumour tissue can aid in predicting the differences in the stromal microenvironment [28]. Hence, understanding the components of stromal microenvironment might be of great help in prognosticating the tumours and customizing therapies.

Studies on TME and its associated stromal components are well established in most epithelial cancers [23, 29]. The presence of this stromal component in the epithelial cancers is expected due to the virtue of the primary tissue that undergoes a transformation. On the other hand, as commented by Cobrinik, for neural cancer like RB, the malignancy is understood to arise from the cone

photoreceptors of the retina [30] that contains an exceptionally high density of microvessels. The tumour development thus occurs conjointly with vascular development in the retina. During the developmental stages, these retinal vasculatures undergo extensive remodelling and maturation, which is reported to contribute to the invasive abilities of tumour cells [31].

The neural tissue, unlike the epithelial tissue, is devoid of stromal components, and hence, it has been difficult to establish the presence or a role for a stromal tissue in RB. In addition to the cell of origin and observed loss of RB function, it is now being recognized that the retinal microenvironment contributes extensively to tumourigenesis [32]. When looking at the presence of stromal cellular subtypes in RB, reports suggest the retinal glial cells (both normal retina and tumour-derived) [32], tumour-associated macrophages (TAMs) and pericytes [33, 34] contribute to RB tumourigenesis by promoting cell proliferation and survival. Findings of cultured tumourspheres and not adherent stromal cells showing RB1 mutation also supported the existence of distinct tumour and stromal components in RB [35]. These existing studies, however, were restricted to the identification of particular cell types such as macrophages and glia and did not provide a holistic view of components in RB TME.

Considering that there is a very little understanding about interactions between tumour cells and non-neoplastic cells in RB, the current study was designed to characterize the cellular TME components seen in RB tumours. This study emphasizes the presence of multiple stromal cell types, which probably by the interaction with RB tumour cells contributes to tumourigenesis. A correlation of stromal cell levels in RB tumours and retina samples would help in a better understanding of the stages of tumour development and disease prognosis. The findings of this study in a broader perspective may be used to tailor novel anti-cancer therapies targeted towards RB tumour stroma or provide decision support in a clinical setting for effective management of RB.

Methodology

Sample Collection and Ethics Approval

The study was reviewed and approved by the local Ethics Committee at Vision Research Foundation, Sankara Nethralaya (Ethics number: 246-2011-P), Chennai, India and the committee deemed that it conformed to the generally accepted principles of research, in accordance with the Helsinki Declaration. This study was exempted from ethics review and was found to comply with the National Statement on Ethical Conduct in Human Research (2007) by the Human

Table 1. Histopathological features of RB tumours used in the study ($n = 12$)

S. No	Age/sex	Laterality	IIRC group	Histopathological features	Invasive status	Chemotherapy details
RB1	2 years/male	OS	D	RB, PD, invasion of the choroid measuring >3 mm, pre-laminar invasion of the optic nerve	Yes	14 cycles post enucleation
RB2	2 years/female	OD	E	RB, WD, choroidal invasion >3 mm, pre-laminar invasion of optic nerve seen	Yes	2 cycles post enucleation
RB3	2.5 Years/male	OS	E	RB, PD, choroidal invasion measuring >3 mm, pre-laminar invasion of optic nerve seen	Yes	NC
RB4	4 years/female	OD	E	RB, PD, choroidal invasion >3 mm, pre-laminar, laminar and post-laminar invasion of optic nerve seen	Yes	6 cycles chemo post enucleation
RB5	1 years/female	OD	E	RB, WD, focal invasion of choroid <3 mm, there is no laminar and post-laminar invasion of optic nerve	Yes	NC
RB6	1.2 years/female	OD	E	RB, WD, focal invasion of choroid <3 mm, pre-laminar invasion of optic nerve seen	Yes	NC
RB7	3 years/male	OS	E	RB, PD, no invasion of choroid. Laminar, pre-laminar and minimal post-laminar invasion of optic nerve	No	NC
RB8	3 years/male	OS	E	RB, WD, no choroidal and optic nerve invasion	No	NC
RB9	5 years/female	OD	D	RB, PD, no choroidal and optic nerve invasion	No	NC
RB10	3 years/male	OS	E	RB, PD, no invasion of choroid, and optic nerve	No	NC
RB11	3 months/ male	OS	E	RB, WD, no invasion of choroid and optic nerve	No	NC
RB12	2 years/male	OS	E	RB, WD, no invasion of choroid, and optic nerve	No	NC

Histopathological features of RB samples used in the study ($n = 12$).

RB, retinoblastoma; IIRC, international intraocular retinoblastoma classification; OS, left eye; OD, right eye; OU, both eyes; PD, poorly differentiated; WD, well differentiated; NC, no chemotherapy; D and E, RB grouping as per IIRC. Chemotherapy using a combination of drugs, namely, carboplatin, etoposide, vincristine and melphalan was administered post enucleation.

Research Ethics Committee at Deakin University (DUHREC approval number 2017-219). Tumour samples were obtained from patients who underwent therapeutic enucleation for RB management. Twelve RB tumours post enucleation received at the L & T Ocular Pathology laboratory between the years 2011 and 2013 were used in the study (histopathological details compiled in Table 1). Informed written consent on the use of these tissues for research purposes was obtained as part of the regular consent during surgery.

Haematoxylin and Eosin (H and E) Staining of RB Tumour Tissue Sections

H and E staining of formalin fixed paraffin embedded (FFPE) sections of RB tumours was performed using standard procedures.

Immunohistochemistry

Immunohistochemistry (IHC) was used for the qualitative estimation of cluster of differentiation (CD)31 (mouse monoclonal antibody, clone JC70A, Dako, Copenhagen, Denmark; endothelial cell – EC marker), CD68 (mouse monoclonal antibody, clone KP1, Pathnsitu Biotechnologies, Hyderabad; TAM marker), alpha

smooth muscle actin (α -SMA; mouse monoclonal antibody, clone 1A4 Dako, Copenhagen, Denmark), (cancer associated fibroblasts [CAFs] marker), vimentin (mouse monoclonal antibody, clone V9, Dako, Copenhagen, Denmark; fibroblasts marker) and synaptophysin (Rabbit monoclonal antibody antibody, clone GR007, Pathnsitu Biotechnologies, Hyderabad; neuroendocrine marker) on FFPE sections of RB tumours ($n = 12$) and associated normal retina. For all the antibodies, the process of antigen retrieval and staining were performed on the Bench Mark GX (Ventana, Roche) automatic devices and antibody positivity was visualised with HRP-conjugated secondary antibodies (Sigma Aldrich). Bound peroxidase was detected with 3,3'-diaminobenzidine (Sigma Aldrich) and counterstained with Mayer's haematoxylin (Sigma Aldrich). Both the central portion of the tumour and pathologically assessed areas of RB tumour cell invasion, namely, the choroid and optic nerve were included in the study. Placental tissue (CD31), reactive lymphoid follicle (CD68), leiomyoma uterus (α -SMA), colon (vimentin) and pancreatic (synaptophysin) tissue sections obtained from SM Surgipath laboratories, Chennai, India, were used as positive controls. All stained specimens were analysed under a light microscope (Nikon Eclipse Ci-L,

Tokyo, Japan) and further reviewed in the presence of 2 pathologists blinded to clinical data. Discrepancies if any were resolved by simultaneous viewing with a multiheaded microscope.

Results

Histopathological Features of RB Tumours (n = 12)

The histopathological features of RB tumours used in the study are summarized in Table 1. Patient cohort consisted of 6 males and 6 females and mean age of the patients was 2.41 years.

H and E Staining Identifies the Existence of Stromal Regions in RB

H and E staining revealed RB tumours to contain both cellular and necrotic areas (Fig. 1a). The cellular areas contained tumour cells arranged as lobules around blood vessels. The vascular channels present in the tumours showed 2 distinct patterns. While the first type of vascular channels did not show extracellular matrix (ECM) depo-

sition (Fig. 1b), the second pattern has deposition of ECM around them (Fig. 1c). The tumour cells showed the invasion of choroid (Fig. 1d) or optic nerve or both. While regions depicted in Fig. 1a–c were seen in all 12 cases analyzed, only RB tumours with invasion ($n = 6$) showed the features represented in Fig. 1d. The diverse stromal cellular subtypes in the RB tumour tissues were further qualitatively explored in the RB tumour sections using IHC.

Expression Pattern of Stromal Markers in RB Tumour and Associated Normal Retina Sections

The expression pattern of stromal markers CD31, CD68, α -SMA, vimentin and synaptophysin was evaluated in FFPE sections of RB tumours (6 invasive and 6 non-invasive cases, histopathological features provided in Table 1) and associated retina sections. The retina sections were microscopically observed to be devoid of tumour cells. Representative IHC images of markers staining in 2 RB tumours, a retina section and positive control are compiled in Figure 2.

Fig. 1. H and E staining reveals the presence of stromal regions in FFPE sections of an invasive RB tumour (Patient-RB1; Table 1). The H and E staining reveals the presence of (a) necrotic areas, (b) tumour lobules with blood vessels without ECM deposition, (c) tumour lobules with central blood vessels and regions of ECM deposition and (d) tumour cells invasion into the choroid. Magnification: 20 $\times$. RB, retinoblastoma; FFPE, formalin fixed paraffin embedded tissue; ECM, extracellular matrix.

Fig. 2. Expression of stromal cellular markers in FFPE sections of RB tumours and retina. Representative images of (a–d) CD 31 staining, (e–h) CD 68 staining (i–l) vimentin staining, (m–p) α -SMA staining, (q–t) synaptophysin in non-invasive RB tumour, invasive RB tumour, retinal tissue section and positive control respectively. Positive controls: placental tissue (d), reactive lymphoid follicular tissue (h), colon tissue (l), leiomyoma uterine tissue (p), pancreatic tissue (t). Magnification: 20 $\times$. CD, cluster of differentiation; TAM, tumour associated macrophages; FFPE, formalin fixed paraffin embedded; α -SMA, alpha smooth muscle actin; CAFs, cancer associated fibroblasts; RB, retinoblastoma.

phoid follicular tissue (h), colon tissue (l), leiomyoma uterine tissue (p), pancreatic tissue (t). Magnification: 20 $\times$. CD, cluster of differentiation; TAM, tumour associated macrophages; FFPE, formalin fixed paraffin embedded; α -SMA, alpha smooth muscle actin; CAFs, cancer associated fibroblasts; RB, retinoblastoma.

(For figure 2 see next page.)

Color version available online

CD31 Expression in RB

The presence of ECs as marked by moderate to strong positivity to CD31 was observed near blood vessels and ECM compartment of RB tumours (Fig. 2a, b). A weak positivity to CD31 was also found in the retina tissue (Fig. 2c). Placental tissue sections were employed as a positive control for immunoreactivity of CD31 antibody (Fig. 2d).

CD68 Expression in RB

TAMs, as identified by the positivity to CD68 marker, were found scattered in the necrotic regions and ECM regions of RB tumours (Fig. 2e, f). The presence of macrophages was also found in the retina tissue section (Fig. 2g). Reactive lymphoid follicular tissue was included as the positive control for anti-CD68 antibody staining (Fig. 2h).

Expression of Vimentin in RB

Moderate to high positivity of vimentin was seen in blood vessels and ECM of RB tumours. Positivity to vimentin is suggestive of the presence of cell types such as fibroblasts, CAFs, mesenchymal stem cells and glial cells (Fig. 2i, j). Retinal tissue also showed a strong positivity to vimentin (Fig. 2k). Colon tissue sections were employed as a positive control for antibody immunoreactivity (Fig. 2l).

α -SMA Expression in RB

Weak to moderate staining of α -SMA suggestive of the presence of fibroblasts and CAFs was observed in RB tumours. The fibroblast population was localized near the blood vessels and the ECM (Fig. 2m, n). Leiomyoma uterine tissue was stained as a positive control for the presence of α -SMA positive cells (Fig. 2p).

Synaptophysin Expression in RB

RB tumour cells showed a strong membrane positivity to synaptophysin (Fig. 2q, r). Positivity of tumour cells to synaptophysin supports the fact that these tumourigenic cells are of neural origin. In contrast to the strong positivity seen in the tumour cells, synaptophysin expression was negative in the stromal areas. The retina also showed positivity to synaptophysin (Fig. 2s). Pancreatic tissue was used as a positive control for the experiment (Fig. 2t).

Taken together, the IHC results for stromal markers in RB FFPE sections revealed the existence of several stromal cell types such as ECs (CD31+), TAMs (CD68+), fibroblasts, mesenchymal stem cells, retinal glia (Vim+), CAFs (α -SMA+). Characteristic distribution of these

stromal cell types was observed in the TME of RB tumours with ECs predominant in the blood vessels and TAMs near necrotic areas or at actively proliferating tumour sites. In all the tumours, fibroblasts were widely distributed with a lesser expression of CAFs. Conversely, synaptophysin expression was confined to the tumour cells of RB and not in the stromal areas. The extent of stromal markers positivity varied between the tumours and did not correlate with histopathological features (invasive or differentiation status) of RB.

Discussion

The role of TME, its associated cellular, non-cellular components have been well studied in detail in various types of adult-onset epithelial cancers [23, 29] and some paediatric cancers such as neuroblastoma and medulloblastoma [17, 36, 37]. Contrarily, very few studies outline the existence of TME in RB [32–35]. The results of the current study confirm the presence of multiple stromal cell types in RB TME akin to the ones observed in other cancer types.

The TME in any tumour comprises rapidly proliferating tumour cells, stromal cells, inflammatory cells, blood vessels and other tissue-associated cells. A study by Houston et al. [38] had earlier detected stromal components of RB TME in a transgenic model of RB, human RB tissue samples and investigated changes in gene expression patterns in different areas of tumours in murine models. These studies emphasized upon the tumour heterogeneity and the TME component further suggesting a focus on stromal components for improving RB tumour management. The current study hence aimed to explore the presence of stromal cell types, namely, ECs, fibroblasts, CAFs and mesenchymal stem cells in RB TME. The presence of each cell type is characterized by positivity to markers [39], the interpretation of marker positivity in RB TME is provided in detail in Table 2.

While the ECs help in the formation of new blood vessels to assist tumour growth and metastasis [40], fibroblasts, CAFs secrete growth factors [41, 42] and contribute to ECM remodelling. The presence of ECs as revealed by positivity to CD31 marker was identified in all the 12 RB tumours assessed, and these cells were majorly found localised in the blood vessels of RB tumours. The positivity to CD31 in the retina could be contributed by the presence of choroidal and retinal ECs. The presence of these ECs in RB stroma is believed to favour tumour growth by supplying abundant growth factors via the blood-retinal

Table 2. Interpretation of stromal cellular components in RB as stained by various IHC markers

Marker used for the study	Positive control	Localization in RB FFPE sections				
		tumour region	RB stromal areas			
			stromal basement membrane like material	fibroblasts, CAFs and muller glial cells	macrophages	endothelial cells
CD31	Placenta	+	–	–	–	+
CD68	Reactive lymphoid follicle	–	–	–	+	–
α -SMA	Leiomyoma uterus	–	–	+	–	+
Vimentin	Colon	–	–	+	–	+
Synaptophysin	Pancreas	+	–	–	–	–

RB, retinoblastoma; IHC, immunohistochemistry; FFPE, formalin fixed paraffin embedded.

barrier. The study also identified the presence of TAMs in all the 12 RB tumours analysed; however, the intensity of positivity varied between tumours. Observed CD 68 positivity in the normal retina is also suggestive of blood-derived macrophages infiltrating the retinal tissue.

On the other hand, fibroblasts, characterized by positivity to vimentin and α -SMA, were the abundant stromal cell type present in RB TME. Positivity to vimentin could also be contributed by the presence of mesenchymal stem cells, macrophages, ECs, pericytes and is suggestive of occurrence of an EMT process [43, 44]. Further, an increased density of CAFs at invasive front is reported to restrict a number of anti-cancer drugs reaching the cell surface [45], and this may be a critical parameter in contributing to drug resistance observed in RB. Analysis of CAF density levels in an additional cohort of drug-resistant tumours would help a better understanding of the role of this cell type in RB drug resistance. Lastly, the positive expression of synaptophysin was confined to RB tumour cells and not stromal areas, and this suggests that RB is a neuroendocrine tumour.

Stromal cell infiltration levels are indicators of disease prognosis and response to therapy. Reports suggest that a strong macrophage signature in TME is known to stimulate angiogenesis [46], the tumour cell survival [47] in turn attributing to poor disease prognosis [48]. Our study results are consistent with these findings and hint an association between increased macrophage infiltration and tumour progression. The mechanisms of disease promotion by TAMs in RB have not been previously explored and warrant further investigation.

The non-neoplastic cells are less susceptible to mutations when compared to the cancerous cells, and molecules selectively expressed on these cells can serve as

attractive targets for therapy. Anti-tumour therapies or drugs targeting TAMs are currently in use for blocking cancer progression [15], and these could also be used for RB management. Earlier studies have identified each RB tumour to behave differently as there exists a difference in regional and temporal difference in gene expression patterns between them [38]. An understanding of underlying TME components is therefore required for the development of novel adjuvant treatments for RB. These multiple cell types seen in RB TME could also contribute to observed angiogenesis and hypoxia, and hence treatment to targeting these stromal cells might provide greater success rates than the ones observed with standard treatment protocols [49]. Given the side effects of systemic chemotherapy and its inhibitory role due to higher tumour stroma [50], wide varieties of local therapies have gained attention in cancer treatment. Reports of better response and fewer side effects with local therapies (intravitreal and intraarterial) have earlier been reported in RB [51–53]. These reports further hint the involvement of stroma in contributing to observed side effects of systemic chemotherapy in RB. The effects of drugs used in local therapies, namely, melphalan on these stromal cells could provide insights on how these drugs affect signalling pathways to result in tumour regression. The findings of the study in a nutshell would open new horizons in defining key events in RB tumour development and response to treatments.

The current study has few limitations; first, about smaller cohort size of RB tumours employed in the study, future quantification studies with increased tumour cohort size would benefit better correlation of the expression patterns of stromal cells to disease staging of patients with RB. At this point, these IHC-based observations do

not provide a clear understanding of how these stromal components work in synergy with the malignant cells and contribute to RB progression. Taking leads from these preliminary findings, in vitro RB models could be established to screen potential candidate markers and decipher the critical signalling pathways mediated by tumour-stroma interactions in RB.

Nevertheless, this current study for the first time has identified the presence of multiple stromal cell types in RB and serves as a proof of concept to establish the fact that RB, like any other epithelial or neural cancer, possesses a TME component. The stromal cellular components of TME along with their vital interactions might contribute to RB progression.

Acknowledgements

Deakin University is acknowledged for providing a scholarship to Ms Rajeswari Raguraman under the Deakin India Research Initiative (DIRI, In country PhD) Graduate student program (code: H960, Student No. 213390445). We are thankful to Prof. Karen Dwyer, Deputy Head, School of Medicine, Deakin University for critically reviewing the manuscript and her valuable suggestions.

Statement of Ethics

Informed consents were obtained from the parents/guardians. The study was approved by Human Ethics Committee at the Vision Research Foundation (Ethics number: 246-2011-P) and granted exemption from ethics review by Deakin University Human Research Ethics Committee (DUHREC: approval number 2017-219).

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

Funding Source

The work was supported with funding received from the Department of Biotechnology, Government of India – grant number BT/01/CEIB/11/V/16.

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