Expression of C-kit in retinoblastoma: a potential therapeutic target

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Background: C-kit is a transmembrane tyrosine kinase protein thought to play an important role in tumourigenesis. With the development of the compound imatinib mesylate, which specifically inhibits tyrosine kinase receptors, C-kit has emerged as a potential therapeutic target. This study aims to determine the immunoexpression of C-kit in retinoblastoma and correlate this expression with histopathological prognostic features.

Methods: Eighty-four paraffin-embedded retinoblastomas were collected from the Henry C Witelson Ocular Pathology Registry. C-kit immunostaining was used according to the protocol provided by Ventana Medical System Inc., Arizona. Immunoreactivity was correlated with the presence or absence of invasion into the choroid and optic nerve and the degree of tumour differentiation. Odds ratios were calculated to quantify differences in C-kit expression between tumours with different patterns of invasion and differentiation.

Results: Twenty-one slides (25%) were excluded from analysis because of the presence of extensive tissue necrosis or the absence of sufficient optic nerve tissue for analysis. Overall, C-kit expression was identified in 33/63 specimens analysed (52.38%). Two of the 13 tumours without choroidal or optic nerve invasion (15.4%) were positive for C-kit. C-kit expression was seen in 31 of the 50 tumours with extraretinal invasion (62%, $p < 0.01$), 26 of 44 specimens with choroidal involvement (59.9%, $p < 0.2$), and 20 of the 29 with optic nerve involvement (68.96%, $p < 0.02$). Fourteen of 25 moderate or well-differentiated specimens (56%) and 19 of 38 undifferentiated specimens (50%) displayed positivity for C-kit ($p > 0.5$).

Conclusions: More than half the retinoblastomas in this study expressed C-kit. The expression of C-kit strongly correlated with histopathological features of a worse prognosis including optic nerve and choroidal invasion.

Retinoblastoma is a rare tumour with an incidence of one in 15 000 to one in 20 000 live births,¹ and represents approximately 4% of childhood cancers.^{1,2} It is the most common primary intraocular malignant tumour of childhood and the most common tumour of the retina.^{1,3} Treatment modalities for retinoblastoma include: enucleation, transpupillary thermotherapy, cryotherapy, laser photocoagulation, chemotherapy, and radiotherapy. The indication for each method depends on the size, location and extension of the disease.³ Over 95% of children with retinoblastoma in the western world will survive this malignancy and 90% will retain vision in at least one eye.^{1,2}

The presence of multiple side effects along with multidrug resistance has, however, fostered the development of a new class of cytotoxic agents. The target of these agents is individual genes that have altered expression in tumour cells.⁴ C-kit (Kit,

CD117, stem cell factor receptor) is a 145 kDa transmembrane tyrosine kinase protein that acts as a type III receptor. The C-kit proto-oncogene, located on chromosome 4q11-21, encodes the C-kit protein, whose ligand is the stem cell factor (SCF; steel factor, kit-ligand, mast cell growth factor). It plays an important role in several major cellular processes such as proliferation, differentiation, apoptosis, attachment and migration.^{4,5} The role of C-kit has been studied in many tumours and overexpression has been seen in many neoplasias.^{5–8} C-kit has emerged as a potential therapeutic target after the recent development of the compound imatinib mesylate (STI571, Gleevec; Novartis Pharma AG, Basel, Switzerland), which specifically inhibits the tyrosine kinase receptors: Bcr-Abl, C-kit and platelet-derived growth factor receptor. The use of imatinib has been approved by the United States Food and Drug Administration to treat KIT-positive gastrointestinal stromal tumours and chronic myelogenous leukaemia.^{5,9}

To the best of our knowledge, only one study has been reported on C-kit in retinoblastoma.¹⁰ The purpose of this article was to study further the immunoexpression of C-kit in retinoblastoma and correlate this expression with histopathological prognostic factors.

MATERIALS AND METHODS

Paraffin blocks

Eighty-four formalin-fixed paraffin-embedded primary retinoblastomas were collected from the archives of the Henry C Witelson Ocular Pathology Laboratory and Registry, McGill University, Montreal, Canada. Age and gender were obtained from histopathological reports.

Immunohistochemistry

Sections were haematoxylin and eosin stained for histopathological assessment. Immunohistochemistry was performed using the Ventana BenchMark LT (Ventana Medical System Inc., Arizona, USA). The fully automated processing of bar code labelled slides included baking of the slides, solvent-free deparaffinisation, and CC1 (Tris/EDTA buffer pH 8.0) antigen retrieval. Slides were incubated with the polyclonal rabbit anti-human C-kit A4502 (DakoCytomation; Dako Canada, Mississauga, Ontario, Canada) at a dilution of 1 : 30 for 30 minutes at 37°C, followed by the application of biotinylated secondary antibody (8 min, 37°C), then an avidin/streptavidin enzyme conjugate complex (8 min, 37°C). Finally, the antibody was detected by Fast Red chromogenic substrate and counterstained with haematoxylin.

For a positive control, sections of skin were used, as C-kit expression is normally present in the melanocytes and some basal cells of skin adnexa.^{6,11} For negative controls the primary antibody was omitted.

Abbreviations: SCF, stem cell factor; TMA, tissue micro-array; VEGF, vascular endothelial growth factor

Table 1 C-kit expression stratified by extent of invasion

	Positive immunoreactivity for C-kit	Negative immunoreactivity for C-kit	Total
	n (%)	n (%)	n
Optic nerve or choroidal invasion	31 (62.00)	19 (38.00)	50
Invasion to optic nerve	20 (68.97)	9 (31.03)	29
Invasion to choroid	26 (59.09)	18 (40.91)	44
Invasion to both optic nerve and choroid	15 (65.22)	8 (34.78)	23
No invasion	2 (15.38)	11 (84.62)	13
Overall	33 (52.38)	30 (47.62)	63

Microscopic classification

Samples were considered positive when more than 30% of the cells presented distinct positive immunostaining. Immunoreactivity was correlated with choroid and optic nerve invasion and the degree of tumour differentiation. Optic nerve invasion was considered present only if tumour cells could be identified beyond the lamina cribosa, and choroidal invasion was considered present when tumour cells were seen to infiltrate through Bruch’s membrane. Minimal choroidal invasion was considered present when tumour cells had destroyed Bruch’s membrane without invading the choroid to depth, with a maximum of three microscopic cell clusters and massive choroidal invasion indicating any choroidal involvement that was not minimal.

The specimens were also classified as well differentiated (presence of Flexner–Wintersteiner rosettes observed in at least 50% of the tumour area), poorly differentiated (no rosettes observed) or moderately differentiated.

Statistical analysis

The chi-squared test was used to assess whether C-kit expression differed with varying extents of tumour invasion and degrees of differentiation. Statistical significance was assumed if the p value was less than 0.05. Odds ratios and corresponding 95% confidence intervals were calculated to quantify differences in C-kit expression between tumours with different patterns of invasion and differentiation.

RESULTS

Eighty-four retinoblastoma specimens were immunostained for this study, of which five (5.95%) were excluded as a result of insufficient viable tumour material. Sixteen (19.05%) were further excluded because of the absence of optic nerve on the slide for analysis. Of the remaining 63 cases (75.00%), the median age at enucleation was 24 months (range 3–84 months) and 27 (42.86%) were from female patients.

Forty-four specimens (69.84%) displayed spread to the choroid and 29 (46.03%) to the optic nerve. Twenty-one

specimens (33.33%) invaded the choroid only, whereas six (9.52%) invaded only the optic nerve. In 23 cases (36.51%) the tumour had spread to both the choroid and optic nerve, and in 13 (20.63%) no spread had occurred beyond the retina. Thirty-six of the 44 specimens with choroidal invasion (81.81%) showed massive invasion, whereas only eight specimens (18.18%) presented minimal invasion of the choroid.

Thirty-eight specimens (60.32%) were poorly differentiated, five (7.94%) were well differentiated, and the remaining 20 (31.75%) were moderately differentiated. Seven specimens (11.11%) displayed an endophytic growth pattern, two (3.17%) were exophytic and the remaining 54 (85.71%) were considered mixed.

Immunohistochemistry

Positive staining for C-kit was observed in 33 of 63 specimens (52.38%). All the positive samples presented cytoplasmatic staining. Among these, it was difficult to determine which also displayed membranous staining. There were no cases with pure membranous staining. There were no significant differences in staining intensity within specimens. As the semiquantitative analysis of staining intensity is highly subjective, samples were classified as only positive or negative (fig 1). No immunostaining of any other ocular structure was seen.

Thirty-one of the 50 tumours with invasion of the optic nerve or choroid (62%) displayed positive immunoreactivity for C-kit compared with only two of the 13 without invasion (15.4%). This increased C-kit expression was shown to be statistically significant.

Similarly, 20 of the 29 tumours with optic nerve invasion (68.97%) displayed positivity for C-kit, compared with only 13 of the 34 specimens with no optic nerve involvement (38.24%). This again proved to be a statistically significant increase in expression.

Whereas the majority of tumours with spread to the choroid were also positive for C-kit, this correlation alone was not statistically significant. As an additional investigation of this relationship, choroidal invasion was further stratified as

Table 2 C-kit expression in tumours with varying extent of invasion

	Invasion to choroid or optic nerve		Invasion to optic nerve		Invasion to choroid			
	Present	Absent	Present	Absent	Present	Absent	Massive	Minimal
Positive immunoreactivity for C-kit	31	2	20	13	26	7	20	6
Negative immunoreactivity for C-kit	19	11	9	21	18	12	16	2
Odds of positive immunoreactivity for C-kit	1.63	0.18	2.22	0.62	1.44	0.58	1.25	3.00
Odds ratio	8.97		3.59		2.48		0.42	
95% Confidence intervals around odds ratio	1.79, 44.95		1.26, 10.23		0.82, 7.51		0.07, 2.35	
Chi-squared p value	<0.01		<0.02		<0.2		<0.5

Figure 1 Graphical representation of C-kit expression stratified by the extent of tumour invasion and differentiation.

“minimal” or “massive”. The extent of choroidal invasion was correlated with the expression of C-kit and this association was not significant (table 1 and table 2, fig 2).

To study the correlation of C-kit and the degree of tumour differentiation, we divided the specimens into two groups. In group 1 we included the moderate and well-differentiated specimens and group 2 was composed only of undifferentiated specimens. Of the 25 specimens in group 1, 14 (56%) displayed positivity for C-kit, whereas of the 38 specimens in group 2, 19 (50%) were positive. This correlation was not statistically significant (see table 3).

DISCUSSION

Protein tyrosine kinases play important roles in cellular mechanisms such as differentiation, proliferation, regulatory processes and signal transduction. As new therapeutic agents are developed, these tyrosine kinases have been subjected to greater attention and are now the focus of many new treatment strategies. Since the introduction of imatinib mesylate, a protein tyrosine kinase inhibitor, C-kit has emerged as an interesting therapeutic target, and expression has been demonstrated in a wide variety of human malignancies.⁸⁻¹²⁻¹⁵

C-kit expression was investigated in more than 120 different tumour types using immunohistochemistry in a tissue micro-array (TMA) format. C-kit was expressed in 28 of 28

gastrointestinal stromal tumours (100%), 42 of 50 seminomas (84%), 34 of 52 adenoid-cystic carcinomas of the salivary gland (65%), 14 of 39 malignant melanomas (35%), and eight of 47 large cell carcinomas of the lung (17%) as well as in 47 additional tumour types.⁶ C-kit expression was also studied in paediatric solid tumours, being observed in 100% of synovial sarcomas, 83% of osteosarcomas, 71% of Ewing tumours, 55% of neuroblastomas, 52% of Wilms' tumours, 77% of embryonal rhabdomyosarcomas and 100% of “rhabdoid” rhabdomyosarcomas. C-kit expression was negative in alveolar soft part sarcomas and desmoplastic small round cell tumours.⁷

In our study, 52.38% of the retinoblastomas expressed C-kit at detectable levels. The choice of antibody used was based on previous studies showing that the polyclonal rabbit anti-human C-kit A4502 (DakoCytomation) was the most sensitive and specific antibody against C-kit, with the least background staining.⁶ We tested several dilutions of this antibody on positive controls and that with the most distinct staining and minimal background positivity was used. As a result of the wide variety of available antibodies, staining protocols with different techniques and scoring criteria, it is often difficult to make comparisons between the different studies investigating C-kit expression in human tumours. Immunohistochemistry results have also been shown to be highly dependent on the specific tissue fixation techniques used by different laboratories.

Figure 2 (A) Detail of Flexner-Wintersteiner rosettes in intra-ocular retinoblastoma displaying positive immunoreactivity for C-kit (original magnification $\times 400$). (B) Invasion at the surgical margin of the optic nerve showing positive expression of C-kit (original magnification $\times 100$).

Table 3 C-kit expression in tumours with varying degrees of differentiation

	Degree of differentiation	
	Moderate + well	Poor
Positive immunoreactivity for C-kit	14	19
Negative immunoreactivity for C-kit	11	19
Odds of positive immunoreactivity for C-kit	1.27	1.00
Odds ratio	1.27	
95% Confidence intervals around odds ratio	0.46, 3.51	
Chi-squared p value	>0.5	

Our rates of positivity were higher than previously reported. Bosch *et al.*¹⁰ used TMA analysis to evaluate C-kit expression in a sample of 73 retinoblastomas and 19.2% displayed positivity for C-kit. Although the same antibody was used as in our work, the dilution was much weaker at 1 : 300. This difference may partly explain the discrepancy between their results and ours. Another explanation would be that the authors used TMA, in which only a small fraction (0.6 mm diameter) of each specimen is used compared with a standard slide. It may well be possible that a particular area of a tumour included in the array was negative whereas other areas would be positive. TMA technology is not appropriate to assess the exact protein expression of an individual case, but instead to take advantage of the large number of samples to profile more general trends related to the tissue type being studied.¹⁶ This is a major limitation of TMA that might explain the lower rates of C-kit expression in their study. Interestingly, C-kit expression is a feature of more aggressive retinoblastomas, with increased expression in tumours spreading beyond the retina.

Retinoblastomas, in the initial stages of development, rely on the retinal vascular network in order to survive and grow. This results in the appearance of "sleeve patterns" of viable cells surrounding blood vessels, with areas of necrosis as the distance from the vessel increases.¹⁷ The current trend in retinoblastoma research indicates that the growth rate of the intra-ocular tumour is more dependent on its ability to induce neovascularisation than on the inherent proliferation rate of the neoplastic cells.¹⁷ The emergence of new blood vessels appears to be an essential step in local invasiveness and systemic spread.^{18, 19}

Angiogenesis is therefore a crucial event in tumour development. Vascular endothelial growth factor (VEGF) is a potent and specific angiogenic growth factor that participates in the formation of the vascular tumour stroma. Kvant *et al.*²⁰ showed that VEGF is hypoxia-inducible in retinoblastoma cells and is also expressed in retinoblastoma paraffin sections. It thus follows that a tumour with a higher angiogenic potential will be more aggressive, and this angiogenic potential is likely to be related to the expression of C-kit by neoplastic cells.

SCF, the ligand for the C-kit receptor, has been implicated in the regulation of neoplastic angiogenesis.²¹ SCF is produced by several types of tumour cells and stimulates mast cell migration, proliferation and degranulation. Mast cells accumulate within and around solid tumours and can release many angiogenic factors, including VEGF.²¹ Activation of C-kit by SCF has a significant effect on VEGF expression, and inhibition of C-kit signalling with imatinib could thus have clinically relevant anti-angiogenic effects.²²

A limitation of our study was the absence of clinical data for the enucleated specimens. As a result we cannot be sure whether any specimen is a primary enucleation, or if chemotherapy or radiotherapy was administered before surgery. From our series it is thus not possible to determine whether conservative treatments have any influence on the expression of C-kit.

The presence of KIT-activating mutations has been documented in a variety of human tumours. Some of them have specific clinicopathological connotations and differ in inhibitor sensitivity. Our paper studied the expression of C-kit in retinoblastoma, and provides convincing evidence that retinoblastoma should be included in the list of C-kit-expressing cancers. Further studies, for example on the in-vitro effect of C-kit inhibition by imatinib mesylate on retinoblastoma cell lines must be undertaken before considering such a drug for the treatment of patients.

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VIDEO REPORT

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Technique of amniotic membrane transplant dressing in the management of acute Stevens-Johnson syndrome

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ABSTRACT

Purpose: To report the technique of amniotic membrane transplantation dressing during the acute phase of Stevens-Johnson syndrome.

Methods: Interventional case report. We report the clinical presentation, surgical technique and clinical outcome of a patient with acute Stevens-Johnson syndrome and progressive tarsal conjunctival epithelial loss.

Results: A 10-year-old girl presented with extensive corneal, bulbar and tarsal conjunctival epithelial loss and severe ocular pain during the acute phase of Stevens-Johnson syndrome. Fornix cleaning and daily rodding was not tolerated, and there was no response to medical drug therapy. Emergency amniotic membrane transplant surgery was performed in both eyes. The conjunctival epithelium recovered completely and the ocular surface remained moist. There was almost complete resolution of ocular pain in the immediate postoperative period. There were no surgical complications and no signs of ocular cicatrisation after 6 months.

Conclusions: In the acute phase of severe Stevens-Johnson syndrome, conservative measures of daily fornix cleaning and rodding may not be practical. AMT dressing may be successfully used to reduce ocular inflammation, promote epithelialisation and prevent ocular cicatrisation.

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