

2005 Galloway Memorial Lecture: Breast Phyllodes Tumours – Morphology and Beyond

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Abstract

Objectives: The aims of this study were to evaluate the predictive role of morphology in a series of breast phyllodes tumours (PTs) in Asian women, and to determine the utility of immunohistochemical expression of p53 and CD117 in the stromal component. **Materials and Methods:** Breast PTs, diagnosed between January 1992 and December 2002 at the Department of Pathology, Singapore General Hospital, were classified into benign, borderline and malignant categories. Surgical margins were evaluated as complete or diffusely involved. Patient follow-up was obtained from casenotes and the Singapore Cancer Registry. Tissue microarrays (TMAs) were constructed using the 2-mm punch on the Beecher arrayer. p53 and CD117 immunohistochemistry was applied on 4- μ m sections cut from TMA blocks. Immunostaining intensity was graded as 0, 1+, 2+ or 3+, for nil, weak, moderate and strong reactivity. The proportion of stromal cells decorated was assessed. Statistical analysis utilised the software SPSS for windows 11.5. Survival curves were plotted using the Kaplan-Meier method, while multivariate analysis was accomplished using the stepwise Cox proportional hazards model. A *P* value of <0.05 was considered a significant result. For verification of protein expression results, a pure stromal population derived from laser capture microdissection was subjected to real-time polymerase chain reaction to determine p53 and CD117 mRNA upregulation. **Results:** Three hundred thirty-five women diagnosed with PT were aged 16 to 69 years (median, 42 years). Tumour size ranged from 0.9 to 25 cm (median, 4 cm). Histologic classification revealed 250 (74.6%) benign, 54 (16.1%) borderline and 31 (9.3%) malignant PTs. Surgical margins were focally involved in 186 (55.5%) cases, diffusely affected in 9 (2.9%) cases and complete in 139 (41.5%) cases. Stromal cells positively stained ranged from 1% to 80% (mean, 15%; median, 5%) for p53, and 1% to 25% for CD117 (mean, 8%; median, 3%). p53 and CD117 staining was associated with PT grade (*P* = 0.004, *P* <0.001). Forty-three (12.8%) women suffered 57 recurrences (mean and median follow-up of 30.3 and 20.4 months respectively). Nine (2.7%) died during follow-up, 7 from malignant disease (mean and median survival duration 37.6 and 23.6 months respectively). Complete margins (*P* = 0.033) resulted in reduced recurrence risk by 51.7%. Though tumour grade was associated with an increased hazard of 1.63, it was not significant (*P* = 0.28). Immunoexpression of p53 was not associated with recurrence (*P* = 0.447), while CD117 was (*P* = 0.001). Upregulation of p53 and CD117 mRNA was found in cases in which there was protein overexpression. **Conclusions:** Involved surgical margins remain a key prognostic parameter in breast PT. Death from disease occurred in women with malignant PTs at presentation, underscoring the need to manage this group more aggressively. p53 staining can be used to corroborate malignancy in PT. CD117 was predictive of recurrence, and if further validated, its expression can be explored for therapeutic purposes. Preliminary molecular studies verify mRNA upregulation in p53 and CD117 overexpressed cases.

Ann Acad Med Singapore 2005;34:671-7

Key words: Grade, Metastasis, Recurrence, Surgical margins

Introduction

The clinical behaviour of phyllodes tumours (PTs) of the breast is difficult to predict.¹⁻⁵ While histological features have been traditionally used as predictors of clinical outcome, specific parameters that define recurrent likelihood

are still not universally agreed upon.² Though grade,^{6,7} stromal overgrowth,^{8,9} tumour necrosis and heterologous stromal elements,¹⁰ or a combination of histologic features,¹¹ have been variously found to be prognostic, a substantial number of reports have concluded that adequacy of surgical

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margins is of paramount importance,^{5,12-15} and that histologic factors have an inconsistent influence on biologic behaviour.

Beyond morphology, investigators have studied the role of biological markers in PT, with p53 perhaps being the most widely evaluated.^{1,14,16-22} p53 expression in stromal cells has been associated with malignant histological features, but does not appear to predict recurrent likelihood.^{1,14,22} Recently, c-kit (CD117) expression was also observed in the stroma of malignant PTs, though its impact on recurrence was not addressed.^{23,24}

In this study, the morphologic features in a series of PTs in Asian women are evaluated. The importance of p53 and CD117 expression in stromal cells was studied using immunohistochemistry on tissue microarrays (TMAs), and the results correlated with histological grade and clinical outcome.

Materials and Methods

*Patients and Tumours*²⁵

Cases of breast PTs, diagnosed between January 1992 and December 2002 at the Department of Pathology, Singapore General Hospital, were histologically reviewed. Patient details and tumour laterality were determined from accession forms, while tumour size was obtained from the surgical pathology reports. Classification into benign, borderline and malignant categories relied on the degree of stromal hypercellularity, cytologic atypia, mitotic activity, stromal overgrowth and nature of the borders (circumscribed versus permeative).¹² Stromal overgrowth was defined as a low power field (x4 microscope objective and x10 eyepiece, 22.902 mm²) that comprised only stroma without epithelial elements.²⁶ A benign PT was diagnosed when the lesion showed pushing margins, mild or moderate stromal hypercellularity, mild or moderate stromal cytologic atypia, occasional mitoses that numbered up to 4 per 10 high power fields, hpf (x40 objective and x10 eyepiece, 0.196 mm²), and no stromal overgrowth. A malignant tumour was defined by marked stromal hypercellularity and cytologic atypia, presence of stromal overgrowth, brisk mitotic activity (more than 10 per 10 hpf), and permeative margins; the finding of a malignant heterologous element placed the tumour into a malignant category. Borderline PTs showed some but not all the characteristics observed in malignant lesions.

Surgical margins, based on available histologic material, were evaluated as complete, when sections sampled showed a surrounding rim of non-lesional breast tissue; focally involved, when tumour extended in only one focus to the inked or cauterised surgical margin; or diffusely involved, when the surgical margin was breached by tumour in more than one focus. In women who underwent further follow-up surgery, surgical margin status was assessed in the latter pathologic specimen.

Patient follow-up was obtained from casenotes and the Singapore Cancer Registry, National Disease Registries Office.

*Tissue Microarray Construction*²⁷

The arrays were constructed with the 2-mm punch on the Beecher arrayer.²⁸ The array layout in grid format was designed using Microsoft Excel. Haematoxylin and eosin (H&E) stained sections of PTs were reviewed and the area of interest marked out on the slide. Using a marker pen, the corresponding region was circled on the archival “donor” paraffin block. The samples were then arrayed on to a “recipient” blank block. Difficulties encountered in the construction of the TMA were overcome as previously described.

*Immunohistochemistry*²⁷

4- μ m sections were cut from the TMA blocks and fished onto coated slides (POLYSINE, Menzel-glaser), which were then baked in the oven overnight at 55°C to enhance adhesion of the sections to the slides. Deparaffinisation in xylene and through graded alcohols followed. For the p53 antibody, sections were subjected to pressurised superheating in Milestone T/T Mega for 4 min at 120°C in 0.01M citrate buffer, cooled for 4 min under running water, then stained with the p53 antibody (D07, Dako M7001) using the Envision ChemMate Kit on the Dako Autostainer. For CD117, sections underwent pressurised superheating in Milestone T/T Mega for 4 min at 115°C in Dako S3307 pH9 buffer, then cooled for 4 min under running water, followed by staining with the CD117 antibody (c-kit, Dako A4502) using the Envision ChemMate Kit on the Dako Autostainer. After that, sections were counter-stained with haematoxylin, dehydrated and mounted in depex. A positive control was run with each staining batch.

Immunostaining intensity was graded as 0, 1+, 2+ or 3+, for nil, weak, moderate and strong reactivity respectively. The proportion of stromal cells decorated was semi-quantitatively assessed as a percentage of the total number of stromal cells present in the microarray. p53 immunostaining was assessed in the nuclei of stromal cells, while CD117 was evaluated in the stromal cytoplasm and cytoplasmic membrane.

Statistical Analysis

Statistical analysis utilised the software SPSS for windows release 11.5. Recurrences were correlated with PT grade, surgical margin status, p53 and CD117 immunostaining results using the χ^2 test. The Mann-Whitney U test was applied to compare means between variables. Disease free and overall survival (DFS, OS) durations were calculated from dates of pathologic diagnosis to the dates of documented histological recurrence and death respectively.

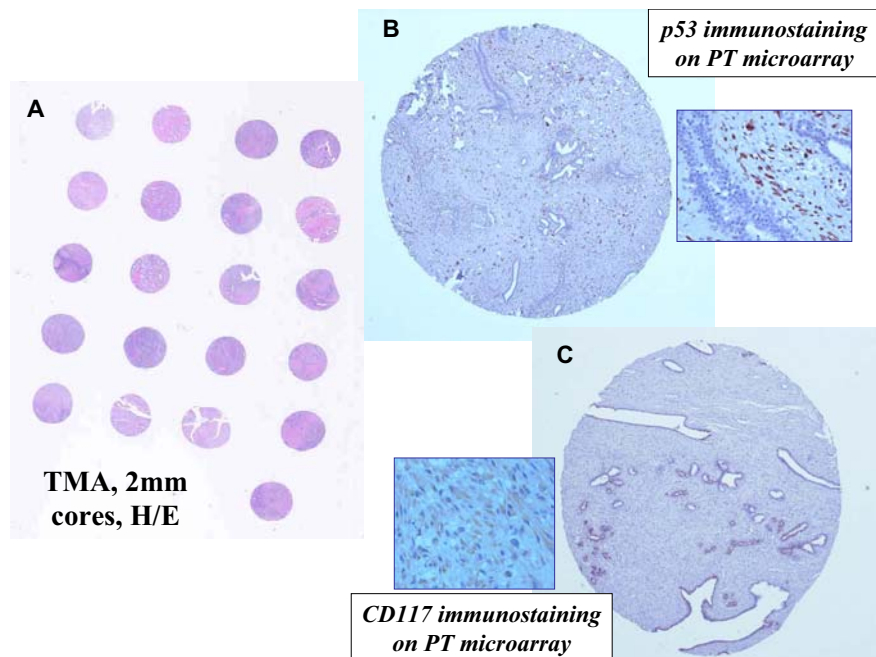


Fig. 1. A. Tissue microarrays of breast phyllodes tumour (PT). B. p53 immunostaining within the stromal cell nuclei of a tissue microarray of a PT. C. CD117 immunoreactivity in the cytoplasm of stromal cells.

Survival time was available for only 206 cases, which was used in the univariate and multivariate survival analyses. Univariate survival curves were plotted using the Kaplan-Meier method, and statistical differences were determined by the log rank test. A *P* value of <0.05 was considered a significant result. Multivariate analysis was accomplished using the stepwise Cox proportional hazards model.

Preliminary Molecular Studies

Laser capture microdissection was used to obtain a pure stromal cell population, which was then subjected to real-time polymerase chain reaction (RT-PCR) using primers to p53 and CD117. Total RNA was extracted from appropriately processed sections cut from the paraffin block, followed by cDNA synthesis from RNA, which was then subjected to RT-PCR. β -actin was used for normalisation.

Results

*Patients and Tumours*²⁵

A total of 335 women were diagnosed with PT during the study period, with ages ranging from 16 to 69 years (mean, 41 years; median, 42 years).

The right and left breasts were affected in 168 (50.1%) and 165 (49.3%) cases, respectively, with 2 (0.6%) women

having bilateral tumours. The microscopic features of the bilateral tumours were almost identical in each case, and for statistical analysis, the right tumours were used. Tumour size for the series ranged from 0.9 to 25 cm (mean, 5.4 cm; median, 4 cm). Histologic classification revealed 250 (74.6%) benign, 54 (16.1%) borderline and 31 (9.3%) malignant PTs. Mitotic activity ranged from nil to 80 mitoses per 10 hpf (mean, 4.45; median, 2) with mean mitotic rates of 1.91, 7.59 and 19.52 mitoses per 10 hpf for benign, borderline and malignant PTs, respectively.

Surgical margins were focally involved in 186 (55.5%) cases, diffusely affected in 9 (2.9%) cases, and considered complete in the remaining 139 (41.5%) cases.

*p53 and CD117 Stromal Immunoexpression*²⁷

The number of microarrays suitable for evaluation of p53 and CD117 immunostaining on stromal components was 289 and 273, respectively. Table 1 shows the distribution of both p53 and CD117 immunohistochemical results, including the staining intensities, in histologically benign, borderline and malignant PTs. The proportion of stromal cells positively stained ranged from 1% to 80% (mean, 15%; median, 5%) for p53, and from 1% to 25% for CD117 (mean, 8%; median, 3%) (Fig. 1).

Positive p53 staining of stromal cell nuclei was associated

Table 1. Association of p53 and CD117 Stromal Immunostaining with Grade of Phyllodes Tumours²⁷

p53 stromal immunohistochemistry	Benign No. (%)	Borderline No. (%)	Malignant No. (%)	Total	<i>P</i>
Negative	150 (81.5)	24 (13)	10 (5.4)	184	0.004*
Positive	69 (65.7)	20 (19)	16 (15.2)	105	
Total	219 (75.8)	44 (15.2)	26 (9)	289	
CD117 stromal immunohistochemistry					
Negative	199 (77.7)	37 (14.5)	20 (7.8)	256	<0.001*
Positive	7 (41.2)	4 (23.5)	6 (35.3)	17	
Total	206 (75.5)	41 (15)	26 (9.5)	273	

* statistically significant values

Table 2. Clinicopathologic Features of Phyllodes Tumour Correlated Against Recurrence²⁵

Clinicopathologic parameter	No recurrence	Recurrence	<i>P</i>
Age			
Mean (y)	41.01	40.88	0.947
Grade/Classification			
Benign	225 (90%)	25 (10%)	0.028*
Borderline	43 (79.6%)	11 (20.4%)	
Malignant	24 (77.4%)	7 (22.6%)	
Tumour size			
Mean (mm)	53.07	60.57	0.285
Mitotic activity			
0-4 per 10 hpf	219 (89%)	27 (11%)	0.184
5-9 per 10 hpf	44 (80%)	11 (20%)	
10 or more per 10 hpf	29 (85.3%)	5 (14.7%)	
Stromal atypia			
Mild	218 (89.3%)	26 (10.7%)	0.016*
Moderate	59 (85.5%)	10 (14.5%)	
Marked	15 (68.2%)	7 (31.8%)	
Microscopic borders			
Pushing/circumscribed	206 (90%)	23 (10%)	0.021*
Permeative	86 (81.1%)	20 (18.9%)	
Stromal hypercellularity			
Mild	160 (91.4%)	15 (8.6%)	0.046*
Moderate	104 (81.9%)	23 (18.1%)	
Severe	28 (84.8%)	5 (15.2%)	
Stromal overgrowth			
Absent	255 (87.3%)	37 (12.7%)	0.808
Present	37 (86%)	6 (14%)	
Margin status			
Focal involvement	160 (86%)	26 (14%)	0.104
Diffuse involvement	6 (66.7%)	3 (33.3%)	
Complete excision	125 (89.9%)	14 (10.1%)	

* Statistically significant result

with PT grade ($P = 0.004$), as was the intensity of staining ($P = 0.002$) whereby a larger proportion of borderline and malignant tumours were more intensely stained. For CD117, positive stromal immunoreactivity was also correlated with tumour grade ($P < 0.001$).

Recurrences and Survival²⁵

A total of 43 (12.8%) women suffered 57 recurrences during the follow-up period (mean and median follow-up duration of 30.3 and 20.4 months respectively). Table 2 shows the relationship between clinicopathologic parameters and onset of first local recurrence. Ethnicity and laterality were not associated with recurrent disease. Nine (20.9%) of the 43 women who experienced recurrences suffered a 2nd recurrence; 4 (9.3%) presented with a 3rd recurrence; while 1 (2.3%) woman recurred a 4th time. All these represented local recurrences, except for 2 (4.7% of women with recurrent disease) with malignant disease who suffered spinal and lung metastases during their 3rd and 2nd recurrent episodes respectively. Histologically, metastases were composed of only malignant stromal elements; no epithelial component was identified.

Nine (2.7%) patients, all but 1 of whom were diagnosed with malignant PT at presentation, died during the follow-up period. The cause of death was from malignant disease in 7 women (mean and median survival duration 37.6 and 23.6 months respectively), while 1 woman diagnosed with benign PT died of carcinoma of unknown origin, and another woman with malignant phyllodes passed away from acute myocardial infarction. There was histological documentation of locally recurrent disease in 6 of these women, followed by metastases (2 histologically documented, the rest radiologically confirmed) that preceded death. As the numbers of borderline and malignant cases that recurred were small, these were grouped together for statistical analysis. The DFS curves for benign (mean, 103 months) and borderline/malignant (mean, 89 months) showed a difference, though not reaching statistical significance ($P = 0.21$). In the overall survival (OS) curves, women harbouring benign PT survived for a mean duration of 142 months, while those with malignant lesions survived for mean and median periods of 87 and 112 months, respectively ($P < 0.01$). No deaths occurred among the borderline cases. The sharp drop for the OS curve represented

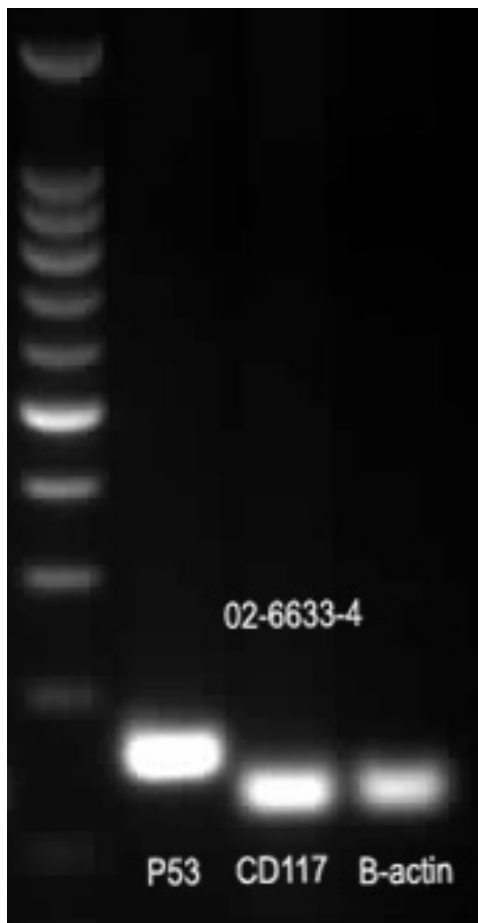


Fig. 2. Real-time PCR showing intense bands corresponding to p53 and CD117.

by benign PT was due to 1 death among this group from cancer of unknown origin.

In the multivariate Cox analysis using stepwise regression, complete margins ($P=0.033$) showed significant prognostic information, with the risk of recurrence being reduced by 51.7% compared to those with focal/diffusely involved margins (relative risk, 0.483; 95% confidence interval, 0.25 to 0.92). Though tumour grade was associated with an increased hazard of 1.63, it was not significant ($P=0.28$). Histologic features of stromal atypia, stromal hypercellularity and microscopic margins, which were statistically significantly associated with recurrence when tested individually, were not significant at the level of the multivariate analysis.

Correlation of p53 and CD117 Stromal Positivity with Recurrent Disease²⁷

Immunohistochemical positivity of p53 in stromal cells was not associated with recurrence ($P=0.447$), while a greater proportion of recurrent tumours demonstrated CD117 stromal positivity ($P=0.001$).

Preliminary RT-PCR Findings

Figure 2 shows the PCR products in a case of PT which had been subjected to laser capture microdissection, with the intense bands corresponding to p53 and CD117, verifying upregulation.

Discussion

The morphologic classification of PTs of the breast is still not ideal. Though histologic grade is used to guide therapeutic strategies, many authors have concluded that it is ultimately the adequacy of surgical margins, rather than the microscopic features, that is predictive of clinical behaviour.

In this study, recurrent disease occurred in 10%, 20.4% and 22.6% of benign, borderline and malignant PTs, respectively. Borderline and malignant tumours recurred within a shorter period, and these women also experienced diminished OS. As with the findings of other investigators,^{6,13,14} an involved surgical margin is a key predictor of local recurrence in this cohort, being independently significant on multivariate analysis, with clear or complete margins associated with a reduction in recurrent risk of 51.7%.

It is noteworthy that all but 2 deaths from disease occurred in women diagnosed with malignant PTs at presentation, underscoring the need to manage this group of women more aggressively at the outset, before recurrence and demise occurs. It is also significant that death from disease was preceded by distant metastases in all cases, confirmed radiologically in the majority and histologically in 2 women, indicating metastatic disease as the pre-terminal event. Therefore, if the primary disease can be completely eradicated at outset, the likelihood of metastases and mortality can be diminished.

Biological markers have often been used as adjuncts to morphology in predicting the behaviour of tumours. p53 is a tumour suppressor gene that is widely studied in neoplastic processes. Located on chromosome 17p13.1, it encodes for a 53 kDa nuclear phosphoprotein that is expressed in all normal cells at low levels.²⁹ The wild-type (normal) p53 gene is involved in cell cycle regulation as well as apoptosis. As the half-life of wild-type p53 is short, immunohistochemistry is believed to highlight expression of mutant p53 protein that is more stable with a longer half-life. In breast PT, the role of p53 has been fairly well studied,^{1,16-18,30} with some authors suggesting that it may be of prognostic value,^{16,18,30} while its predictive utility has not been affirmed by others.^{1,17} It has been suggested that p53 immunohistochemical expression in stromal cells can be relied upon as an adjunctive tool in the diagnosis of malignancy.^{19,20} In this study, p53 staining was correlated with PT grade, but did not reveal an association with

recurrent disease, corroborating the conclusions of other authors, and supporting its potential use as a confirmatory marker of malignancy.

CD117, also known as c-kit, is a membrane bound tyrosine kinase receptor whose overexpression is characteristically observed in gastrointestinal stromal tumours.³¹ It has served as a therapeutic target by drugs (Glivec) used to manage patients with these tumours. More recently, CD117 has also been found in the stromal cells of malignant PTs, and it was postulated that its overexpression may be instrumental in the growth of these tumours.²³ In this current study, CD117 was predictive of malignancy and recurrent disease, and if its role in malignancy and recurrence of PT is validated, its expression in these tumours can be advantageously harnessed for therapeutic purposes.

While studies on p53 and c-kit have focused predominantly on protein expression using immunohistochemistry; at the molecular level, sequencing of the p53 gene has been carried out by various authors with mostly mutations being discovered^{16,19,32} though one study disclosed a wild-type gene sequence in a malignant PT.³³ Proliferative activity using Mib-1 and S-phase fraction,^{30,34} microvessel density,³⁵ CD34 and factor VIIIa stromal positivity³⁶ are also believed to be potentially helpful in predicting the behaviour of PTs, though their clinical relevance remains to be further validated.

The use of TMA has revolutionised translational research by allowing the rapid screening of numerous tumour samples simultaneously to validate candidate genes as potential prognostic markers.²⁸ In this study, TMA technology was used to evaluate the immunohistochemical expression of p53 and CD117 in a large series of breast PTs. If standard histological sections were used, the number of sections requiring assessment would incur not only substantially more time, but also additional cost of antibodies and reagents because of the larger sections and greater number of slides. Previous reports reviewing the accuracy and reliability of TMA versus standard sections have concluded that results derived from TMA are representative.^{37,38}

In conclusion, this study represents the largest series to date in the experience of a single institution on breast PTs. The higher incidence of PTs among younger Asian women has been alluded to.^{39,40} In an earlier Singapore report of 40 women from another institution,⁴¹ 60% were under 40 years old, and it was considered that the younger age of presentation may pose a problem in accurate preoperative diagnosis. In this study, there did not seem to be a predominance of younger individuals among the 335 women, with 42% under 40 years of age. During the same period of the study from 1992 to 2002, 4842 cases of breast carcinoma were diagnosed at the Department of Pathology,

Singapore General Hospital. When comparing these 2 tumours, the number of PTs would form 6.9% of the total cases of breast cancers, though it has to be noted that these 2 tumours are distinct and not directly comparable. This figure is higher than the 0.5% to 1.5% quoted,^{8,12} and may reflect a truly greater frequency of this tumour among Asian women. Re-visiting clinicopathologic parameters reveal that careful and thorough appraisal of light microscopic morphology is still of importance in the evaluation of breast PTs, with histologic grade together with its constituent elements of stromal atypia, microscopic borders and stromal hypercellularity being correlated with recurrence; while surgical margin status is independently prognostic on multivariate analysis. Protein expression of p53 and CD117 within the stroma shows a correlation with grade, and may therefore be used as an ancillary method to corroborate a diagnosis of malignancy. Preliminary molecular studies using paraffin embedded material and laser capture microdissection verify mRNA upregulation of p53 and CD117 within stromal cells. The role of CD117 in recurrent disease requires more investigation, and holds promise as a potential therapeutic target in clinical management.

Acknowledgements

Singapore Cancer Registry, National Disease Registries Office, for follow-up information.

This study was supported by grants from the Department of Clinical Research, Singapore General Hospital and the Singapore Cancer Syndicate (SCS-MS0004).

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