Heterogenous expression of ERG oncoprotein in Malaysian men with adenocarcinoma of the prostate

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Abstract

Introduction: Prostate cancer is a heterogenous disease and the mechanisms that drive it to behave differently are not well understood. Tumour expression of the ERG oncogene occurs in the majority of patients with prostate cancer in Western studies. This is considered to be oncogenic as ERG acts as a transcription factor to regulate genes involved in tumour proliferation and invasion. In this study we investigated expression of ERG in Malaysian men with prostate cancer. Methods: Tissues were collected from 80 patients with clinically detected prostate cancer and treated with radical prostatectomy. Cases were tested for ERG by immunohistochemistry using the mouse monoclonal antibody EP111. All blocks on 48 cases were tested in order to determine the extent of heterogeneity of ERG expression within individual cases. ERG expression was analysed in relation to patient age, ethnicity and tumour stage and grade. Results: Forty-six percent of cases were ERG positive. There was no significant association between ERG and tumour grade or stage. Sixty-nine percent of Indian patients had ERG positive tumours; this was significantly higher (p=0.031) than for Chinese (40%) and Malay (44%) patients. Heterogeneity of ERG expression, in which both positive and negative clones were present, was seen in 35% of evaluated cases. Evaluation by tumour foci showed younger patients had more ERG positive tumour foci than older patients (p=0.01). Indian patients were more likely to have the majority of tumour foci with ERG staining positively, compared to either Chinese or Malay patients (P <0.01). Conclusion: In this study, tumour expression of ERG was more likely to occur in patients of Indian ethnicity.

Keywords: TMPRSS2-ERG, ERG, ethnic variation

INTRODUCTION

A fundamental goal of cancer research is to identify key mutations in oncogenes which are responsible for driving a tumour into an aggressive cancer that will both invade adjacent tissues locally and metastasize. This is particularly important for prostate cancer as it is well known that a large numbers of screen detected prostate cancers are relatively indolent and unlikely to result in mortality.¹ In addition, prostate cancer is notoriously heterogeneous with respect to exhibiting different clones of cancer within the same prostate gland; some of higher grade and potentially more aggressive than other clones.² Consequently, the diagnosis and management of prostate cancer is particularly challenging, as in order to be truly effective, a prognostic test is needed that can reliably differentiate the aggressive tumour foci from the indolent ones. To date, no test is able to achieve this with a high degree of accuracy; consequently there is considerable over-treatment of indolent prostate cancer, by either radical prostatectomy or radiotherapy.³ Both these treatments can result in unwanted side effects following treatment, such as urinary incontinence and erectile dysfunction, which adversely affect the quality of life of men diagnosed with prostate cancer.

A key oncogenic event occurring in up to sixty-percent of prostate cancers in western
cohnets is the fusion of the androgen driven
TMPRSS2 gene to the proto-oncogene ERG.\textsuperscript{4} ERG protein expression is then greatly increased
due to androgen binding to the promoter region
of the TMPRSS2-ERG fusion. This increased
ERG expression is considered to be oncogenic
as it acts as a transcription factor for the
downstream regulation of a number of genes,
known to be important in tumour proliferation
and invasion.\textsuperscript{5} It has been shown in numerous
studies comparing ERG protein expression to
TMPRSS2-ERG gene fusion that up regulation of
ERG in tissue samples is caused predominantly
by the TMPRSS2-ERG fusion event, and this
over expression of ERG can be reliably detected
using immunohistochemistry.\textsuperscript{5-9}

There is still considerable debate about
whether or not over-expression of ERG in
prostate cancer is a marker of aggressive tumours
and therefore of poor prognosis.\textsuperscript{4, 10-12} However, in
the prostate it does appear to be only associated
with invasive adenocarcinoma of the prostate and
its pre-cursor lesion, prostatic intra-epithelial
neoplasia (PIN).\textsuperscript{13} Interestingly, there is evidence
to suggest that the expression of ERG occurs
more frequently in western studies of prostate
cancer\textsuperscript{13,14} than it does in Asia, where in some
cancer cohorts its frequency of occurrence has
been reported to be as low as thirty-percent.\textsuperscript{15-20}

Epidemiological studies of men with prostate
cancer show distinct differences in terms of
clinical incidence and mortality rates between
ethnic groups.\textsuperscript{21} While prostate cancer is the
most frequently diagnosed male malignancy in
the Western world including Northern Europe,
the USA and Canada, it is notably less common
in Asian countries\textsuperscript{21, 22} despite a rapid increase in
recent years.\textsuperscript{21-23} Specifically, in Malaysia despite
having a relatively lower incidence in comparison
to Western countries, the incidence of prostate
cancer has increased by twenty-percent between
the years 2007 to 2011. It is also noted that about
40\% of men with prostate cancer have metastatic
disease at presentation,\textsuperscript{24} unlike western studies
where the majority of cases are detected at an
earlier stage. Among the major ethnic groups in
Malaysia, the Chinese have the highest incidence
of prostate cancer (nine-percent), followed by
Malaysian Indian (six-percent) and Malaysian
Malay (five-percent).\textsuperscript{25}

In the current study we seek to investigate
the expression of ERG in a retrospective series
of prostate cancers from Malaysian men with
clinically detected hormone naïve prostate cancer
treated by radical prostatectomy at a large city
hospital. If future research establishes ERG
as a key event promoting aggressive forms of
prostate cancer, it is important to determine its
relevance to Asian men. Moreover, examining the
uniformity of ERG expression between different
tumour foci in each radical prostatectomy, will
help determine whether ERG expression is a
main driving event in these Asian men with
prostate cancer.

**MATERIALS AND METHODS**

**Tissue samples**

Tissue samples were retrospectively collected
from clinically detected prostate cancer patients
who were treated with radical prostatectomy at
Hospital Kuala Lumpur, Malaysia from 2007 to
2013. A total of 80 cases were selected based
on the availability of samples for review. Whole
prostatectomy samples were fixed in neutral
buffered formalin for 24 hours before processing
to paraffin wax and embedding. For the study,
the Haematoxylin & Eosin (H&E) stained
slides for each tissue block were reviewed and
samples showing invasive adenocarcinoma were
selected by a pathologist for testing. Patients’ data
including age and ethnicity were collected for all
cases where available from the patient’s medical
record folder. Histological type and tumour
grading according to the Gleason’s grading
system were determined from the pathologist’s
report. The American Joint Committee on Cancer
(AJCC Cancer) Prostate Cancer Staging Manual
8\textsuperscript{th} edition was used for pathological tumour
staging.\textsuperscript{26} On 48 of the total 80 cases, all available
blocks for each case and containing tumour were
tested for ERG expression; on the remaining 32
cases, tissue blocks containing tumour with the
primary Gleason grade were chosen for testing.
Permission for the analysis of human tissue was
obtained from the Medical Research & Ethics
Committee, Malaysian Ministry of Health
(NMRR-10-1400-7968).

**Immunohistochemical testing for ERG
 Oncoprotein Expression**

Tissue sections were cut at 4 micrometers using a
rotary microtome and the sections mounted onto
Superfrost Plus Slides (Thermo Scientific, USA)
for maximum adhesion. Immunohistochemical
detection of ERG oncoprotein expression was
achieved using the previously validated\textsuperscript{4} ERG
monoclonal antibody Clone EP111 (Dako,
Denmark). Briefly, the paraffin wax embedded
sections were dewaxed in xylene and rehydrated
in a series of graded alcohols before antigen
retrieval in Tris EDTA (pH 9) for 30 minutes at 100°C. The sections were then treated with 0.3% H₂O₂ to block endogenous peroxidase for 10 minutes. Incubation with the ERG mAb (Clone EP111, Dako, Denmark) diluted at 1:100 was carried out for 1 hour at room temperature. Primary antibody was detected using DAKO REAL EnVision Detection System with a horse radish peroxidase label (Dako, Denmark) for 1 hour at room temperature and visualised using 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen (Dako, Denmark). Nuclei were counterstained with Harris’s haematoxylin (Leica, Germany) for 1 minute. The expression of ERG oncoprotein was evaluated microscopically and recorded as positive when the tumour nuclei stained positively, regardless of the proportion of tumour cells stained or the staining intensity. Experimental runs contained negative controls in which the primary antibody was omitted whilst the staining of vascular endothelial cells of small vessels functioned as the internal positive control.

In addition, a tissue section from a prostatic adenocarcinoma known to stain strongly for ERG was included in each staining run. A case, comprising a number of different blocks, was considered to be positive if at least one tumour block showed positivity for ERG expression.

Statistical analysis
Statistical analyses were performed using SPSS 16.0 software for Windows (IBM, Inc., New York, NY, USA). Clinical and pathological features of the cases were compared across groups of patients using frequencies and percentages. The difference of distribution of clinical and pathological characteristics across different ethnicity groups were evaluated using the Chi square test. Results were considered to be significant if the P-value was less than 0.05.

RESULTS
The median age of the patients was 67.0 years (range, 53-78 years). The majority of the patients comprised Chinese (50.0%), followed by Malay (33.8%) and Indian ethnicities (16.3%) (Table 1).

ERG expression
Overall, 37/80 (46.3%) of the cases were positive for ERG expression (Fig. 1). With respect to ethnicity, 9 out of 13 Indian patients (69.2%) had ERG positive tumours; this proportion is significantly higher (p=0.031) than in the other two ethnicities, with 16/40 (40.0%) of Chinese patients and 12/27 (44.4%) of Malay patients having ERG positive tumours, respectively. ERG was not found to be associated with tumour grade or stage (Table 2).

Heterogeneity for ERG oncoprotein expression, in which both ERG positive and negative clones were present, was seen in 35.4% (17/48) of cases, in which all tissue blocks were tested. Evaluation by tumour foci showed that patients younger than the median age of 67 years had more ERG positive tumour foci than older patients (p=0.01). Similarly, Indian patients were not only more likely to have ERG positive tumours than the other two ethnicities, but when positive for ERG were also more likely to have the majority of tumour foci staining (P<0.01) (Table 3, and Fig. 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chinese</th>
<th>Malay</th>
<th>Indian</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at Surgery</td>
<td></td>
<td></td>
<td></td>
<td>0.262</td>
</tr>
<tr>
<td>Median (Range)</td>
<td>67 (54-77)</td>
<td>64 (54-78)</td>
<td>66 (53-73)</td>
<td></td>
</tr>
<tr>
<td>Gleason Score - N (%)</td>
<td>0.812</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7 (8.75)</td>
<td>3 (3.75)</td>
<td>2 (2.50)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>27 (33.75)</td>
<td>17 (21.25)</td>
<td>8 (10)</td>
<td></td>
</tr>
<tr>
<td>8 or above</td>
<td>6 (7.50)</td>
<td>7 (8.75)</td>
<td>3 (3.75)</td>
<td></td>
</tr>
<tr>
<td>Pathological T Stage - N (%)</td>
<td>0.817</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>27 (33.75)</td>
<td>16 (20)</td>
<td>8 (10)</td>
<td></td>
</tr>
<tr>
<td>T3a*, b</td>
<td>13 (16.25)</td>
<td>11 (13.75)</td>
<td>5 (6.25)</td>
<td></td>
</tr>
</tbody>
</table>

*PT3a tumours comprised 5 cases only.
Malaysia comprised the 3 most populous ethnicities in Asia; Malay, Chinese and Indians. Therefore, with respect to its people and its cultures it represents a microcosm of Asia. In comparing the expression of the ERG oncogene in men with prostate cancer from different Asian ethnicities, our study has the advantage that the men all attended the same medical center. Therefore, the processing and testing of the tissue samples was standardised and uniform throughout. This is not necessarily the case when comparing the results from different studies, conducted at different centres and in different countries.

Prostate cancer is notoriously multifocal. Morphologic and molecular analysis carried out in the past has demonstrated that up to eighty percent of prostates can harbour multiple separate cancers by the time of diagnosis.²⁻²⁰

**DISCUSSION**

TABLE 2: Evaluation of the association of ERG oncoprotein expression status with clinical and pathological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ERG Negative</th>
<th>ERG Positive</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Total N</td>
<td>43</td>
<td>54</td>
<td>37</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;67</td>
<td>18</td>
<td>49</td>
<td>19</td>
</tr>
<tr>
<td>≥67</td>
<td>25</td>
<td>58</td>
<td>18</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>24</td>
<td>60</td>
<td>16</td>
</tr>
<tr>
<td>Malay</td>
<td>15</td>
<td>56</td>
<td>12</td>
</tr>
<tr>
<td>Indian</td>
<td>4</td>
<td>31</td>
<td>9</td>
</tr>
<tr>
<td>Path T Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>59</td>
<td>21</td>
</tr>
<tr>
<td>3a*, b</td>
<td>13</td>
<td>45</td>
<td>16</td>
</tr>
<tr>
<td>Gleason Score</td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>7</td>
<td>58</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>56</td>
<td>23</td>
</tr>
<tr>
<td>≥8</td>
<td>7</td>
<td>44</td>
<td>9</td>
</tr>
</tbody>
</table>

*PT3a tumours comprised 5 cases only.

TABLE 3: Evaluation of heterogeneity of ERG expression in all tissue blocks of 48 cases of prostate cancer and association with patient age and ethnicity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ERG Negative</th>
<th>ERG Positive</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Total N</td>
<td>149</td>
<td>67</td>
<td>73</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;67</td>
<td>58</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>≥67</td>
<td>91</td>
<td>74.6</td>
<td>31</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>69</td>
<td>87.3</td>
<td>10</td>
</tr>
<tr>
<td>Malay</td>
<td>63</td>
<td>65.6</td>
<td>33</td>
</tr>
<tr>
<td>Indian</td>
<td>17</td>
<td>36.2</td>
<td>30</td>
</tr>
</tbody>
</table>
FIG. 1: Immunohistochemical staining for the expression of truncated oncoprotein erythroblast transformation-specific-related gene (ERG). Representative images demonstrating: (A) The endothelial cells of small vessels showed positive endogenous ERG expression in the context of surrounding ERG-negative tumour. (B, C, D) Nuclear staining of the tumour cells is apparent in ERG positive tumours; original magnification x200.

FIG. 2: Inter-focal heterogeneity of ERG oncoprotein expression in different tissue blocks from one patient. Blocks A, B, C, E and F exhibited ERG oncoprotein expression with intense nuclear staining; (D) Absence of ERG staining in the tumour but positive endogenous ERG expression on the endothelial cells can be seen; original magnification x200.
Previous studies have also reported inter-focal and intra-focal variability in ERG protein levels in prostate cancer. Therefore, we considered it important to our investigations to systematically evaluate the question of heterogeneity of ERG expression in the samples. Overall, we observed a high rate (35%) of inter-focal heterogeneity for ERG oncoprotein expression in our cases.

Similar to other studies investigating the frequency of ERG expression in radical prostatectomy patients, in comparison to Asian countries, the overall frequency of ERG over-expression in our study was 46.3%. Studies from the west, especially from the United States and Europe have shown slightly higher TMPRSS2-ERG fusion and ERG expression rates (>50%) in comparison to Asian countries with some studies showing less than 30% of cases exhibiting TMPRSS2-ERG fusion or ERG expression. Interestingly, we observed significant differences in ERG expression in the prostate cancer of Malaysian men. As with a recent study on TRUS biopsies, we found expression of ERG to be more common in the radical prostatectomy samples of Indian Malaysian men (69%), than Malay (44%) or Chinese Malaysian men (40%) with prostate cancer. Moreover, when occurring in the tissue samples of Indian men, ERG expression tended to occur in the majority of the tumour foci i.e. there was less heterogeneity of expression; suggesting that ERG is associated with the main driver of prostate cancer in these cases. This is in contrast to ERG expression in the prostatectomy samples of Chinese and Malay Malaysian men, where it occurred less frequently and when it did occur tended to be in a minority of the tumour foci (tissue blocks). Whilst the numbers are small, these are important observations as it suggests that an oncogenic event other than ERG expression, is the main driver of prostate cancer in Chinese and Malay men. This is particularly relevant in Malaysia where the data shows prostate cancer to occur more commonly in Chinese men, than the other two main Malaysian ethnicities.

The other main observation was that ERG expression occurred more frequently in samples of patients less than the median age of 67 years of age, and was significantly associated with greater uniformity of expression i.e. when ERG positive, the majority of foci in the radical prostatectomy were positive. These findings are in agreement with studies that have similarly demonstrated an association of TMPRSS2 ERG fusion status with younger age at diagnosis.

Future studies are required to establish the prognostic relevance of ERG in prostate cancer. However, a number of studies to date have shown an association of ERG expression with worse prognosis indicated by higher tumour stage and metastasis or tumour-specific mortality. In addition, there is a need to determine the events responsible for the occurrence of the fusion gene, such as the activity of the androgen receptor, and how this in turn may reflect ethnic differences in ERG expression in prostate cancer. These events are likely to involve both genetic and environmental factors.

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