Quantitation of bcl-2 protein in bladder cancer tissue by enzyme immunoassay: comparison with Western blot and immunohistochemistry

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Apoptosis (programmed cell death) and the genes regulating this process (e.g., bcl-2), have recently become a focus of interest in the study of cancer development and progression. We adapted and evaluated a new enzyme immunoassay method (EIA) for quantifying bcl-2 in cell lysates. The range of detection of the assay was 5–400 kilounits/L with inter- and intraassay CVs of 5.5–9.2% and 5.0–8.8%, respectively. The recovery of added bcl-2 protein to cell lysates was 96–104%. The concordance rates with Western blotting and immunohistochemistry were 97.5% and 93.7%, respectively. Bcl-2 concentrations were measured in the cell lysate of bladder tumors. The amount of bcl-2 in 158 bladder cancer (mean rank, 71.3 kilounits/g protein; range, 8.4–324 kilounits/g protein), was significantly higher than in nondiseased bladder tissues distant to the tumors (mean rank, 31.5 kilounits/g protein; range, 5–54.9 kilounits/g protein), \( P = 0.00001 \). Bcl-2 expression was correlated to tumor proliferative capacity, which was measured by DNA flow cytometry as the percentage of cells in the synthetic phase of the cell cycle. The enzyme immunoassay provides a rapid, quantitative, and reliable technique for measurement of bcl-2 in tumor tissue. The detection of substantial amounts of bcl-2 in invasive tumors (compared with nondiseased tissues) suggests that the assay should be a useful tool for investigating the prognostic value of bcl-2 in bladder tumors and for selecting patients for future anti-bcl-2 therapy.

Programmed cell death is known to play an important role in the cellular response to genotoxic stress; thus, loss of apoptotic response in tumor cells is thought to be one of the mechanisms involved in malignant progression and resistance to chemotherapy (1).

The bcl-2 gene product is supposed to contribute to oncogenesis by suppressing signals that induce apoptotic cell death. Several studies have shown overexpression of bcl-2 protein in a variety of solid tumors, including prostatic carcinoma (2), colorectal cancer (3), squamous cell carcinoma (SCC)³ of the lung (4), breast cancer (5, 6), and nasopharyngeal malignancies (7). In the literature, bcl-2 expression has been reported only in transitional cell carcinoma of urinary bladder (TCC) (8–12).

Studies of bcl-2 in solid tumors have been conducted primarily by immunohistochemical and/or molecular techniques (2–16). These methods, although important in investigating the biological characteristics of these tumors, are subjective and cumbersome for clinical application. In contrast, the enzyme immunoassay (EIA) method allows for rapid quantitation and objective assessment of this variable for clinical purposes.

In this study, we validate and evaluate an EIA method to quantify bcl-2 in bladder tissue and compare the results with Western blot (WB) and immunohistochemistry (IHC). We correlate the results with clinopathologic factors and tumor proliferative capacity [fraction of cells in the S-phase of the cycle (SPF) measured by DNA flow cytometry] to evaluate their potential clinical importance.

**Materials and Methods**

**Tissue specimens and patients**

All the tissue specimens analyzed in the study were obtained from surgical transurethral biopsy or resection and cystectomy of 158 patients with bladder cancer attending Ain Shams Hospitals in Cairo. Forty-four samples were obtained from women, whose median age was 50 years (range, 24–70 years), and 114 samples were ob-

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³ Nonstandard abbreviations: SCC, squamous cell carcinoma; TCC, transitional cell carcinoma; EIA, enzyme immunoassay; WB, Western blot; IHC, immunohistochemistry; and SPF, S-phase fraction.
tained from men, whose median age was 61 years (range, 26–77 years). Biopsies from 40 of these patients were also collected from the nondeased tumor-distant bladder tissue (informed consent was obtained from all subjects). The biopsies were divided into three equal portions: One portion was processed for histopathological evaluation, the second portion was processed for DNA flow cytometry analysis; and the third portion was cytologically confirmed by touch imprints and then rapidly frozen and stored at −80 °C for quantitation of bcl-2.

In all the cases, diagnosis was based on clinical signs of hematuria, urinary cytology, and conventional pelvic ultrasound scan. On the basis of histological diagnosis, tumor biopsies were classified as TCC (102 of 158) or SCC (56 of 158), and schistosomiasis was present in 48.1% of the tumors. The neoplastic tissues were moderate- to well-differentiated carcinomas, and staging was performed according to the tumor-node-metastasis pathological staging system.

CHEMICALS AND REAGENTS
Acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate, N,N,N',N'-tetramethylethylenediamine, low molecular weight marker proteins, aprotinin, phenylmethylsulfonyl fluoride, β-mercaptoethanol, benzamidine, diithiothreitol, Tris, coomassie blue G, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma Chemical Co. The nitrocellulose membrane was from Bio-Rad. Mouse monoclonal antibody clone 124 against bcl-2, rabbit anti-mouse IgG alkaline phosphatase conjugate, biotinylated rabbit antimouse antibody, the avidin-biotin-peroxidase complex antibody clone 124 against bcl-2, rabbit anti-mouse IgG, alkaline phosphatase conjugate, biotinylated rabbit anti- mouse antibody, the avidin-biotin-peroxidase complex kit, and dianinobenzidine were purchased from Dako. Propidium iodide stain (Coulter DNA-Prep Reagent Kit, containing 50 mg/L propidium iodide, 4000 kU/L bovine pancreas type III RNase, 1g/L NaN3, saline, and stabilizers) was obtained from the Coulter Corp. The bcl-2 EIA kit was purchased from Endogen.

SAMPLE PREPARATION FOR FLOW CYTOMETRY
Fresh tissue samples were mechanically dissociated, and cell suspensions were processed for DNA analysis as previously described (17).

FLOW CYTOMETRY
Flow cytometric analysis was performed with a Coulter EPICS Profile II flow cytometer, configured with a 488 nm argon ion laser. Stained tonsillar lymphocytes were used as a diploid control. Twenty thousand events per sample were acquired and analyzed with MultiCycle software (Phoenix Flow Systems). A modified exponential debris function was used to subtract the debris in the DNA histograms. The SPF was defined as the proportion of cells in the DNA histogram with intermediate DNA content between that of G0/G1 and G2/M.

PREPARATION OF CELL lysates AND MEASUREMENT OF BCL-2 IN CELL lysates BY EIA
All steps of sample preparation were devised in our laboratory. All steps were carried out at 4 °C. Tissues were washed in ice-cold saline and homogenized on ice in 10 mmol/L HEPES buffer (pH 7.5; containing 10 mmol/L K2EDTA, 50 mmol/L NaCl, 5 mmol/L benzamidine, 10 mmol/L Triton X-100, 10 mmol/L β-mercaptoethanol, 0.39 mmol/L phenylmethylsulfonyl fluoride, and 5 mg/L aprotinin) with an Ultraturrax T-25 homogenizer for three bursts of 60 s each, separated by a pause for 1 min. The homogenate was incubated in the lysing buffer on ice for 30 min, with vortex-mixing every 10 min. The homogenate was then centrifuged at 20 000g for 20 min with a Beckman L7 ultracentrifuge at 4 °C, and the resulting supernatants (lysates) were frozen at −80 °C before use. We quantified the protein concentration in lysates by the Bradford method (18), using bovine serum albumin as the calibrator.

Bcl-2 antigen was measured in cell lysates with adjusted protein concentrations (1 g/L) with a monoclonal antibody-based EIA (Endogen), according to the manufacturer’s instructions. Briefly, the anti-bcl-2 fluorescein isothiocyanate conjugate was applied to a microtitre plate coated with a mouse monoclonal antibody specific to human bcl-2 protein and then incubated with cell lysates, as well as with the manufacturer-supplied kit calibrators (human bcl-2 antigen at 0–400 kilounits/L). The resulting immune complexes were bound onto the plate, and any unbound reactants were removed by a washing step. Next, the wells with the bound immune complexes were incubated with a linking solution (horse radish peroxidase-labeled sheep anti-fluorescein isothiocyanate IgG) and then with a substrate-chromogen solution (3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide) to develop color. The color development step was stopped by the addition of 0.18 mol/L sulfuric acid. The intensity of color developed was read spectrophotometrically at 450 nm vs a substrate blank.

Bcl-2 was expressed in units, which were defined as the amount of bcl-2 protein in 1000 lysed cells of an internal control cell line.

DETECTION OF BCL-2 BY WB TECHNIQUE
WBs were performed according to Sambrook et al. (19) and adapted by us as follows. Sixty micrograms of cell lysate proteins in loading buffer (50 mmol/L Tris, 20 g/L sodium dodecyl sulfate, 100 mL/L glycerol, 100 mmol/L dithiothreitol, pH 6.8) were boiled for 3 min and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were transblotted to nitrocellulose filters in Tris-glycine buffer (25 mmol/L Tris, 192 mmol/L glycine, 200 mL/L methanol, pH 7.4) for 5 h at 60 V. The nitrocellulose sheets were washed, and unoccupied binding sites were saturated with 50 g/L bovine serum albumin in Tris-buffered saline buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1 mL/L

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NP-40) for 1 h at room temperature. Then the filters were sequentially incubated with phosphate-buffered saline supplemented with 10 g/L bovine serum albumin and a mouse monoclonal anti-bcl-2 antibody diluted 1:300 (by volume) overnight at 4 °C, then with rabbit anti-mouse IgG alkaline phosphatase conjugate diluted 1:500 (by volume) for 90 min at room temperature. Each of these steps was separated by 3–5 min washes in phosphate-buffered saline, containing 3 mL/L Tween 20. Finally, the filters were incubated with alkaline phosphatase substrate solution (1 mL of substrate buffer, containing 100 mmol/L NaCl, 5 mmol/L MgCl₂, 100 mmol/L Tris, pH 9.5, with 4 μL of nitro blue tetrazolium and 4 μL of 5-bromo-4-chloro-3-indolyl phosphate) at room temperature until the developed bands were of the desired intensity, then the reaction was stopped by 200 μL of 0.5 mol/L EDTA (pH 8) and 50 mL of phosphate-buffered saline. Comparison of the resulting nitrocellulose with others in which normal mouse IgG serum was substituted for bcl-2 monoclonal antibody permitted the identification of bcl-2 band.

**IMMUNOHISTOCHEMICAL STAINING**

Samples were fixed in 100 mL/L buffered formalin for <30 h, paraffin blocks were prepared, 4 μm sections were mounted on poly-lysine-coated slides and incubated at 37 °C for 18 h. The sections were deparaffinized, and endogenous peroxidase activity was quenched with a 30 mL/L hydrogen peroxide solution in methanol. Sections were dehydrated, and antigen unmasking using the microwave was performed. Sections were placed into 10 mmol/L citrate buffer (pH 6) and microwaved at a power setting of 750 W for 15 min, with subsequent heating at 450 W for 10 min (20). After they were cooled, the sections were incubated with primary mouse monoclonal anti-bcl-2 antibody at 1:20 dilution for 60 min at room temperature. Then they were incubated with secondary biotinylated rabbit anti-mouse antibody at a dilution of 1:100 with 50 mL/L heat-inactivated human serum in 50 mmol/L Tris buffer (pH 7.6). The sections were then incubated with avidin-biotin-peroxidase complex. Diaminobenzidine was used as the chromogen, with hematoxylin as the counterstain.

**STATISTICAL ANALYSIS**

The Mann–Whitney and Kruskal–Wallis nonparametric tests and χ² test were performed for comparison of bcl-2 expression between various groups. Spearman correlation analysis was used to correlate bcl-2 and SPF and bcl-2 and grades. For all analyses, two-sided tests of significance were performed. All analyses were performed using the statistical package for the social sciences (SPSS) on an IBM personal computer.

**Results**

**EIA PERFORMANCE CHARACTERISTICS**

**Precision.** We tested the precision of the assay by measuring three tissue lysate pools six times in one assay (within-run) and in five consecutive assays (between-run). The results are shown in Table 1. Within- and between-run CVs ranged from 5% to 8.8% and 5.5% to 9.2%, respectively.

**Lower detection limit.** The lower detection limit of this assay was 5 kilounits/L, which is the concentration corresponding to a signal 3 SD above the mean of a zero calibrator.

**Analytical recovery.** In experiments on the analytical recovery of bcl-2, we used three lysate pools (10.3, 50.2, and 110.4 kilounits/L). We assayed each sample in duplicate after addition of three different amounts of bcl-2 manufacturer-supplied kit calibrators (82, 43, and 25 kilounits/L). The calculated recovery range was 96% to 104% of added bcl-2 (Table 2).

**CUTOFF POINTS FOR SPF AND BCL-2**

A cutoff point of 10% for the SPF was calculated from results obtained from nondiseased tissues (mean SPF ± 2 SD) and on the basis of previous reports (21). For bcl-2 protein, 60 kilounits/g protein was calculated as the best
cutoff point that discriminates between high proliferative tumors (SPF >10%) and low proliferative tumors (SPF ≤10%), Table 3. Correlation coefficient analysis between the SPF and bcl-2 revealed significant correlation, at $r = 0.56$ and $P = 0.01$.

COMPARISON OF BCL-2 EXPRESSION BY WB AND EIA
Seventy-nine specimens were analyzed by both WB and EIA. Seventy-seven of 79 (97.5%) of these specimens showed agreement between the two methods (Fig. 1). However, there was one specimen that was negative by EIA and positive by WB, as well as one specimen that was positive by EIA and negative by WB (Table 4 and Fig. 2). For the purpose of this comparison, a cutoff value of 60 kilounits/g protein was used.

COMPARISON OF BCL-2 EXPRESSION BY IHC AND EIA
Seventy-nine specimens were analyzed by both IHC and EIA for comparative purposes (Fig. 3). The concordance was 93.7%. There were four samples that were negative by IHC but positive by EIA ($≥60$ kilounits/g protein). In addition, there was one sample negative by EIA ($<60$ kilounits/g protein) but positive by IHC (Table 5 and Fig. 4).

### Table 3. Sensitivity and specificity at different cutoff points of bcl-2 for discriminating between low (SPF ≤10%) and high proliferative (SPF >10%) bladder tumors.

<table>
<thead>
<tr>
<th>Cutoff, kilounits/g protein</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥50</td>
<td>70.5</td>
<td>77.1</td>
</tr>
<tr>
<td>≥55</td>
<td>68.2</td>
<td>82.9</td>
</tr>
<tr>
<td>≥60*</td>
<td>68.2</td>
<td>88.6</td>
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<tr>
<td>≥65</td>
<td>63.4</td>
<td>88.6</td>
</tr>
<tr>
<td>≥70</td>
<td>56.8</td>
<td>91.4</td>
</tr>
</tbody>
</table>

*60 kilounits/g protein is the cutoff that maximizes the sum of sensitivity and specificity.

### Table 4. Comparison of bcl-2 expression by Western blot and tissue lysate EIA.

<table>
<thead>
<tr>
<th></th>
<th>Western blot</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>31</td>
</tr>
</tbody>
</table>

* Concordance, 97.5%.

CLINICAL SAMPLE ANALYSIS
Bcl-2 concentrations were measured by EIA in the lysate of bladder carcinoma as well as in nondiseased bladder tissues (Fig. 5). Bcl-2 values ranged from 8.4 to 324 kilounits/g protein (mean rank, 71.3 kilounits/g protein) for tissues obtained from 158 carcinomas and 5–54.9 kilounits/g protein (mean rank, 31.5 kilounits/g protein) when the assay was done on 40 nondiseased tissue samples. The difference between the two groups was statistically significant, at $z = 5.58$, $P = 0.00001$. In our study, the frequency distribution of bcl-2 concentrations in bladder tumors vs nondiseased tissues revealed that bcl-2 concentrations $≥60$ kilounits/g protein were measured in 43% of bladder carcinoma specimens but in none of nondiseased bladder tissues, suggesting that bcl-2 concentrations $≥60$ kilounits/g protein may correspond to bcl-2 overexpression. With regard to clinicopathological factors, bcl-2 expression was not related to tumor-node-metastasis staging, histologic type, or the presence of schistosomiasis (Table 6). However, bcl-2 concentration was correlated to the histologic grade of the tumor: Poorly differentiated tumors had higher bcl-2 concentrations than lower grade tumors.

### Discussion
The hypothesis that altered pathways of cell death may contribute to the very early stage of disease has been
suggested. In particular, bcl-2 overexpression was shown to be a frequent molecular event involved in the first stage of bladder carcinogenesis (8).

This is the first study to quantify the basal concentration of bcl-2 in TCC and SCC of the urinary bladder and to establish a cutoff value, which may be of clinical importance to clarify bcl-2 interaction in the process of bladder tumorigenesis and its impact on therapy and clinical outcome. In this study, we have tried to develop an easier and less time-consuming technique that would enable us to have cutoff values for bcl-2 assayed in cell lysates. The performance of the bcl-2 EIA has been evaluated and been shown to be reliable for the quantitation of bcl-2 in minimal amounts of cell lysates (50-μL samples of cell lysates at a protein concentration of 1 g/L) prepared from tissues obtained from patients with bladder cancer. Measurement of the bcl-2 antigen in specimens was confirmed by both WB and IHC for selected samples. Concordance between the EIA and these more standard

### Table 5. Comparison of bcl-2 expression by immunohistochemical staining and EIA.

<table>
<thead>
<tr>
<th>Immunohistochemical staining</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2 EIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>29</td>
<td>10</td>
</tr>
</tbody>
</table>

*Concordance, 93.7%.*
methodologies suggests that the EIA can be used reliably in place of WB and/or IHC. From a technical perspective, this option offers greater ease in performing an analysis for bcl-2 in tissue lysates. In addition, the EIA offers the advantage of quantitatively determining the bcl-2 concentration as opposed to IHC and WB methods, which tend to be more subjective measures of bcl-2 expression. The 6.7% discordance between EIA and IHC observed in our study may be explained either by the loss of antigenicity due to the fixation of tissue for IHC or by the use of different pieces of tissue from a tumor for the separate methodologies. On the other hand, only 2.5% disagreement between EIA and WB was shown because both methods used the same cell lysate.

The presence of bcl-2 protein has not been reported in schistosomal bladder cancer. However, bcl-2 expression was studied only in TCC. By IHC, bcl-2 was found in 17.8–24% of invasive TCC (10, 11). We detected bcl-2 overexpression in 43% of bladder cancers: 43% of TCCs, 42.9% of SCCs, 48.7% of samples associated with schistosomiasis, and 37.8% in absence of schistosomiasis. Indeed, bladder cancer is thought to be a multistep disease in which various molecular alterations interact (22). Apoptosis may represent a mechanism of elimination of cells that have acquired such genetic alterations and are more predisposed to abnormal proliferation. Furthermore, cells unable to undergo apoptosis may be more susceptible to the accumulation of genetic alterations than nondiseased cells. Because healthy bladder tissues express low bcl-2 concentrations, we can hypothesize that altered expression of bcl-2, and the consequent block of apoptotic pathways, may represent a first step in bladder carcinogenesis.

The substantial increase of bcl-2 among the cancer cells of poorly differentiated bladder tumors points to a potentially critical role of this apoptosis suppressor protein in bladder cancer progression. Overexpression of bcl-2 may serve as a determinant of an advantageous cell survival in bladder tumor cell populations, ultimately leading to tumor progression and metastasis, as evident in the present study by the significant correlation between bcl-2 overexpression and tumor proliferative capacity (measured by DNA flow cytometry as the percentage of cells in the synthetic phase of the cell cycle).

Bcl-2 is considered to be a new chemoresistance gene, because its expression protects the cells from apoptotic cell death induced by different anticancer drugs. An antisense approach to reduce the concentrations of bcl-2 protein and overcome chemoresistance has been successfully tried in relapsing non-Hodgkin lymphoma (23), and additional investigations of bcl-2 antitherapy in other types of tumors, including bladder cancer, are encouraged.

In conclusion, our data indicate that bcl-2 expression can reliably be measured in bladder cancer by the EIA method. The substantial increase of bcl-2 in poorly differentiated and high proliferative bladder tumors suggests a potential role of bcl-2 in bladder cancer progression. Moreover, bcl-2 antisense, if administered before chemotherapy in these patients, may have a sensitizing effect, and monitoring the concentration of bcl-2 protein in response to bcl-2 antisense therapy can be easily done by the bcl-2 EIA method.

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**References**


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**Table 6. Relationship between histologic type, grade, tumor-node-metastasis classification, presence of schistosomiasis, and bcl-2 expression in bladder cancer.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number</th>
<th>Bcl-2, kilounits/g protein</th>
<th>Bcl-2 &lt;60 kilounits/g protein</th>
<th>Bcl-2 ≥60 kilounits/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean rank</td>
<td>Median</td>
<td>Mean rank</td>
</tr>
<tr>
<td>Stage Ta-T1</td>
<td>34</td>
<td>40.2</td>
<td>61.5</td>
<td>20</td>
</tr>
<tr>
<td>Stage T2</td>
<td>34</td>
<td>46.8</td>
<td>59.7</td>
<td>16</td>
</tr>
<tr>
<td>Stage T3</td>
<td>90</td>
<td>37.4</td>
<td>64.7</td>
<td>54</td>
</tr>
<tr>
<td>Stage N0</td>
<td>138</td>
<td>13.5</td>
<td>54.4</td>
<td>79</td>
</tr>
<tr>
<td>Stage N+</td>
<td>20</td>
<td>18.4</td>
<td>64.1</td>
<td>11</td>
</tr>
<tr>
<td>Grade 1</td>
<td>24</td>
<td>25.4*</td>
<td>35.8</td>
<td>20*</td>
</tr>
<tr>
<td>Grade 2</td>
<td>88</td>
<td>36.1</td>
<td>48.2</td>
<td>52</td>
</tr>
<tr>
<td>Grade 3</td>
<td>46</td>
<td>48.2</td>
<td>60.7</td>
<td>18</td>
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<tr>
<td>TCC</td>
<td>102</td>
<td>68.7</td>
<td>67.7</td>
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<tr>
<td>SCC</td>
<td>56</td>
<td>76</td>
<td>65.2</td>
<td>32</td>
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<tr>
<td>Schistosoma +</td>
<td>76</td>
<td>78.4</td>
<td>66.2</td>
<td>39</td>
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<tr>
<td>Schistosoma –</td>
<td>82</td>
<td>65.4</td>
<td>63.1</td>
<td>51</td>
</tr>
</tbody>
</table>

*Statistically significant difference between different grades by: a Kruskal–Wallis analysis at $\chi^2 = 7.7$, $P = 0.02$; and b $\chi^2$ test at $\chi^2 = 7.1$, $P = 0.02$. 

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