A Study of p53 Protein and DNA Ploidy in Retinoblastoma

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Abstract

Background: Retinoblastoma is the most common primary intraocular cancer in children. It arises from cells that are defective in both copies of the retinoblastoma susceptibility gene (RB1). Loss of both RB1 and p53 functions may be required for cell immortalization and tumor development. The pattern of p53 expression in retinoblastoma appears to depend on the state of differentiation of the tumor; p53 and RB pocket proteins are important to control DNA ploidy, which may have a value in estimating the prognosis of retinoblastoma.

Purpose: The aim of this work was to assess p53 expression, DNA ploidy, and their relations with histopathologic features in retinoblastoma cases.

Subjects and Methods: The study was done on eight patients with retinoblastoma (seven males and one female). Personal and family history with pedigree analysis were done for all cases. Ophthalmic examination with follow up of tumor regression rate during therapy was performed. Eight primary retinoblastoma samples were obtained from enucleated eyes of all patients. Retinoblastoma sections were stained by hematoxylin and eosin stain and scoring of histopathologic features in retinoblastoma cases.

DNA ploidy was examined by assessment of type of DNA histogram and DNA index using cytomeric analysis system (CAS 200) after feulgen staining. The proliferative activity was automatically expressed by the CAS 200 as the percentage of cells engaged in the S-phase of the cycle.

Results: Our results indicated that p53 protein was immunohistochemically detectable in most retinoblastoma cases (7/8 cases), and was only negative in one case. DNA was aneuploid in 6 out of 8 cases, while 2 cases (one of them was p53 negative, and the other showed weak positivity) were diploid with high proliferative activity. Histopathologic examination revealed that 3 cases were poorly differentiated and 5 cases showed intermediate differentiation with increased necrotic changes and mitotic figures.

Conclusion: Retinoblastoma samples showed high degree of p53 protein expression and high degree of aneuploidy which were related to the aggressiveness of histopathologic changes of retinoblastoma. Thus both p53 expression and DNA ploidy have been shown to be markers of aggressiveness of tumor behaviour in retinoblastoma and can help in the prediction of its prognosis.

Key Words: Retinoblastoma – p53 – DNA ploidy.

Introduction

RETINOBLASTOMA (RB) is the most common primary intraocular cancer in children with 3-7 cases per million people per year worldwide [1]. Histologically, the tumor often consists of primitive small round cells with hyperchromatic nuclei and scanty cytoplasm, and characterized by formation of rosettes (Flexner Wintersteines (FW) rosettes) [2]. Retinoblastoma arises from cells that are defective in both copies of the retinoblastoma susceptibility gene (RB1). Most retinoblastoma tumor cells eventually undergo programmed cell death (i.e. apoptosis); however, some cells can acquire the ability to metastasize and become immortal. Transfection of immortal retinoblastoma cells with DNA sequence encoding wild-type p53 protein induces cell death, suggesting that the loss of both RB1 and p53 functions may be required for cell immortalization and tumor development [3]. Mutation of p53 usually leads to an abnormal protein with a markedly extended half-life, resulting in accumulation of this product, which can be detected histochemically [4]. The pattern of p53 expression in retinoblastoma appears to depend on the state of differentiation of the tumor, which reflects the developmental stage of retinoblasts [5]. The steadily increasing interest in DNA ploidy determination of malignant tumors led to an increased application of flow and image cytomeric methods in tumor research for some years. Investigations are mainly designed to explore the proliferative capacity and deviations in the characteristic DNA content and to obtain clues about the actual malignancy grade and the prognostic outcome of a given malignant tumor therapy. Because chromosomal aneuploidy...
has been accepted as an early key event in tumorigenesis caused by genetic instability, the cytometric equivalent of chromosomal aneuploidy detected by DNA image cytometry (DNA-ICM) may serve as a marker of neoplasia [6].

The aim of this work was to assess p53 expression, DNA ploidy, and their relations with histopathologic features in retinoblastoma cases.

**Subjects and Methods**

This study was carried out on eight patients with retinoblastoma (7 males & 1 female). Patients were examined in the Eye Tumor Unit, Ophthalmic Department, Ain Shams University, during a period from February 2002 to January 2004. Their ages ranged from 1.5 to 5 years. Personal and family history with pedigree analysis were done for all cases. Ophthalmic examination with follow up of tumor regression rate during therapy was performed. Eight primary retinoblastoma samples were obtained from enucleated eyes of all patients. Samples were formalin fixed, paraffin embedded and five micron sections were prepared for routine hematoxylin and eosin stain, immunostaining for p53 expression, and feulgen stain for DNA analysis.

**Histopathology:**

Scoring of the histopathologic features was performed according to Schouten-van Meeteren et al. (2001) [2]. Differentiation was classified by percentage of Flexner Wintersteines (FW) rosette formation, a tumor was judged as well differentiated if more than 80% of the area showed rosettes, and poorly differentiated if rosettes were absent, with remaining samples classified as intermediate. Necrosis was graded according to the percentage of the necrotic tumor area. The number of mitotic figures was classified according to the number of mitoses per high power field (HPF): Non (fewer than 1/10 HPF), some (more than 1/10 HPF), clear (more than 5/10 HPF).

**Immunostaining for P53:**

Sections for immunohistochemistry were stained using the avidin-biotin-peroxidase complex (ABC) method. Sections from a breast cancer known to have p53 over expression served as positive controls. Negative controls in the form of mouse IgG1 control antibody was used.

**Evaluation of Immunostaining:**

p53 immunostaining was assessed quantitatively by microscopic examination using a scoring system for both intensity and extent of staining according to Teh et al. (2002) [7]. For intensity 0, 1, 2 and 3 were used for no, weak, moderate and strong staining respectively. For extent of staining 0, 1, 2 and 3 were used for no nuclear staining, lesser than 10%, 10-50% and greater than 50% nuclear staining respectively. The score for intensity and extent of staining were summed for each case. A score of at least 4 represented cases with marked staining for p53, 2 & 3 for moderate staining, 1 for weak staining and 0 for negative staining.

**Feulgen Stain for DNA Analysis:**

The tissue sections were feulgen stained using the cytomeric analysis system (CAS) quantitative DNA staining kit.

**DNA Analysis & Interpretation of DNA Histogram:**

DNA analysis was performed by the CAS 200 image analyzer. The imaging system was calibrated by measuring the DNA content of at least 20 cells of the control [rat hepatocytes (calibration slides) which were supplied with the feulgen staining kits and stained in the same run with the test slides]. For our studied cases the first 150-200 non overlapped cells were randomly selected for DNA analysis. The DNA histograms were clarified according to Danque et al. (1993) [8] and Dreinhofer et al. (2002) [9] as diploid, tetraploid, and aneuploid based on the amount of DNA relative to the normal control. Diploid histogram is considered when a single peak is found in the diploid region and fewer than 20% of the cells are present in the tetraploid position. Tetraploid type was considered when there is a peak in the diploid region and a second peak with more than 20% of the cells in the tetraploid region. Aneuploid type was considered when at least 10% of the cells show a distinct peak outside the diploid or tetraploid position. The proliferative activity was automatically expressed by the CAS 200 as the percentage of cells engaged in the S-phase of the cycle and was classified according to Masters et al. (1989) [10], into low (<10%), mild (10-20%), moderate (20-30%) and high (>30%). According to Sidoni et al. (1996) [11], DNA histogram was classified as diploid if DNA index was from 0.9 to 1.1 and aneuploid in all other cases.

**Results**

This study included eight patients with retinoblastoma (7 males & 1 female). Their ages ranged from 1.5 to 5 years. Positive parental consanguinity was reported in 37.5% of patients. Non of them had family history of retinoblastoma. History, clinical data, management, and follow up of retinoblastoma cases are summarized in table (1). The histopathologic findings in the examined samples...
are summarized in table (2). Five cases showed intermediate differentiation with the presence of Flexner Wintersteines rosette (FW) formation in less than 80% of the tumor samples (Fig. 1). The other 3 cases were poorly differentiated with absence of FW rosettes. The necrotic changes of the tumor was <30% in 2 samples while one case showed 30-50% necrotic changes and the residual 5 samples showed necrotic changes exceeding 50%. Mitotic figures were present in more than 1/10 HPF and fewer than 5/10 HPF in 6 cases (some) while the other 2 cases showed more than five mitotic figures in 10 high power field (>5/10 HPF) (clear). Quantitative p53 immunostaining scores, and DNA ploidy are shown in table (3). DNA analysis revealed that six cases were considered aneuploid. The main cell population in each sample was detected in S-phase and the rest of cells were distributed in the diploid, tetraploid and aneuploid areas with more than 10% of cells detected outside the diploid and tetraploid areas which refer to their high aneuploidy, while two cases were diploid but with high proliferative activity (Figs. 3,4).

Table (1): History, clinical data, and management of retinoblastoma cases.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Father age</th>
<th>Mother age</th>
<th>Family history</th>
<th>Consanguinity</th>
<th>Laterality</th>
<th>Management</th>
<th>Regression rate</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5y</td>
<td>Male</td>
<td>30y</td>
<td>20y</td>
<td>-ve</td>
<td>-ve</td>
<td>Unilateral</td>
<td>Enucleation &amp; orbital implant</td>
<td>Gradual</td>
<td>-ve</td>
</tr>
<tr>
<td>2</td>
<td>1.5y</td>
<td>Male</td>
<td>40y</td>
<td>35y</td>
<td>-ve</td>
<td>+ve</td>
<td>Unilateral</td>
<td>Enucleation &amp; orbital implant</td>
<td>Gradual</td>
<td>-ve</td>
</tr>
<tr>
<td>3</td>
<td>1.5y</td>
<td>Male</td>
<td>25y</td>
<td>20y</td>
<td>-ve</td>
<td>-ve</td>
<td>Unilateral</td>
<td>Enucleation &amp; orbital implant</td>
<td>Gradual</td>
<td>-ve</td>
</tr>
<tr>
<td>4</td>
<td>2.5y</td>
<td>Male</td>
<td>40y</td>
<td>30y</td>
<td>-ve</td>
<td>-ve</td>
<td>Unilateral</td>
<td>Enucleation &amp; orbital implant</td>
<td>Rapid</td>
<td>+ve (Optic nerve infiltration)</td>
</tr>
<tr>
<td>5</td>
<td>2.5y</td>
<td>Male</td>
<td>35y</td>
<td>20y</td>
<td>-ve</td>
<td>+ve</td>
<td>Unilateral</td>
<td>Enucleation &amp; orbital implant</td>
<td>Rapid</td>
<td>-ve</td>
</tr>
<tr>
<td>6</td>
<td>4y</td>
<td>Female</td>
<td>45</td>
<td>30</td>
<td>-ve</td>
<td>+ve</td>
<td>Unilateral</td>
<td>Enucleation &amp; orbital implant</td>
<td>Rapid</td>
<td>-ve</td>
</tr>
<tr>
<td>7</td>
<td>1.5y</td>
<td>Male</td>
<td>30y</td>
<td>20y</td>
<td>-ve</td>
<td>-ve</td>
<td>Unilateral</td>
<td>Enucleation &amp; orbital implant</td>
<td>Rapid</td>
<td>-ve</td>
</tr>
<tr>
<td>8</td>
<td>1.5y</td>
<td>Male</td>
<td>45y</td>
<td>40y</td>
<td>-ve</td>
<td>-ve</td>
<td>Bilateral</td>
<td>Enucleation &amp; orbital implant &amp; chemotherapy</td>
<td>Rapid</td>
<td>-ve</td>
</tr>
</tbody>
</table>

Table (2): Histopathologic features of the examined samples of retinoblastoma.

<table>
<thead>
<tr>
<th>No.</th>
<th>F.W.R.</th>
<th>Necrotic changes</th>
<th>Mitotic figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Absent</td>
<td>&gt;50%</td>
<td>Some</td>
</tr>
<tr>
<td>2</td>
<td>Present</td>
<td>&lt;30%</td>
<td>Some</td>
</tr>
<tr>
<td>3</td>
<td>Present</td>
<td>&gt;50%</td>
<td>Some</td>
</tr>
<tr>
<td>4</td>
<td>Present</td>
<td>&gt;50%</td>
<td>Clear</td>
</tr>
<tr>
<td>5</td>
<td>Absent</td>
<td>&gt;50%</td>
<td>Some</td>
</tr>
<tr>
<td>6</td>
<td>Present</td>
<td>&lt;30%</td>
<td>Some</td>
</tr>
<tr>
<td>7</td>
<td>Present</td>
<td>&gt;50%</td>
<td>Clear</td>
</tr>
<tr>
<td>8</td>
<td>Absent</td>
<td>30-50%</td>
<td>Some</td>
</tr>
</tbody>
</table>

F.W.R. = Flexner Wintersteines rosettes.
Absent = Poor differentiation.
Present = Intermediate differentiation.
Some = >1 mitotic figure/10 HPF.
Clear = >5 mitotic figures/10 HPF.

Table (3): Quantitative p53 immunostaining scores and DNA ploidy.

<table>
<thead>
<tr>
<th>No.</th>
<th>P53 immunostaining</th>
<th>DNA ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marked (score 4)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>2</td>
<td>Moderate (score 2)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>3</td>
<td>Weak (score 1)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>4</td>
<td>Moderate (score 3)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>5</td>
<td>Weak (score 1)</td>
<td>Diploid with high proliferative activity</td>
</tr>
<tr>
<td>6</td>
<td>Weak (score 1)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>7</td>
<td>Marked (score 4)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>8</td>
<td>Negative (score 0)</td>
<td>Diploid with high proliferative activity</td>
</tr>
</tbody>
</table>
A Study of p53 Protein & DNA Ploidy in Retinoblastoma

Discussion

Retinoblastoma (RB) is the most common intraocular malignancy of childhood, occurring in approximately 1 in 20,000 live births [12]. It arises from pluripotent retinal progenitor cells. Mutations of the RB1 gene promote tumor development by deregulating the E2F family of transcription factors leading to uncontrolled cell cycle progression [13].

Eight patients presented with retinoblastoma were examined in this study, seven males and one female, with negative family history of similar cases, and positive parental consanguinity in 37.5% of patients. Many epidemiological studies showed male preponderance in retinoblastoma [14, 15, 16]. On the other hand a twelve year epidemiological review study of RB in Omani children in 2004 showed female preponderance, and also non of them had a positive family history [17].

Histopathologic findings in the examined samples revealed that 3 cases were poorly differentiated and 5 cases showed intermediate differentiation with increased necrotic changes and mitotic figures (3 cases showed more than 50% necrotic changes and 2 cases showed more than 5 mitotic figures/10 HPF). Shields et al. [18], and Schouten-van Meereren et al. [2], stated that patient with poorly differentiated retinoblastoma have a worse prognosis, compared to patients with rosettes present in the tumor.

Apoptotic cell death is prevalent in RB. The ability of exogenous p53 to stimulate cell death in cultured human RB cells suggests that p53 plays a role in regulating cell death in RB [19]. The expression of p53 (detection of p53 protein) in 8 samples of human retinoblastoma was determined by immunohistochemical analysis revealed that there was negative expression of p53 in 1 sample.
Stability of DNA ploidy by cytometric analysis system (CAS) in the present work revealed that 6 cases were aneuploid, while 2 cases (one of them was p53 -ve, and the other showed weak positivity) were diploid. This support the suggestion of the presence of residual or normal functioning p53 gene is important to decrease aneuploidy in retinoblastoma. A high degree of aneuploidy characterizes the majority of human malignancies. Aneuploid status can arise through mitotic or cleavage failure coupled with failure of tetraploid G(1) checkpoint control, or through deregulation of centromere number, thus altering the number of mitotic spindle poles. p53 and RB pocket proteins are important to the control of G(1) progression, and p53 has been also suggested as important to the control of centromere duplication [23]. Carder et al. [24], discussed the role of wild type p53 in preventive genomic instability and stated that this instability appears to result from DNA replication errors and tends to produce aneuploid subclones. Brito et al. [25], detected that tumor with p53 gene overexpression was associated with DNA aneuploidy. Song [26], analyzed paraffin embedded tissue masses of retinoblastoma from 20 patients using flow cytometry (FCM) with the results that the rate of occurrence of DNA aneuploidy was 35% and that the post-operative survival rate of diploid and near diploid tumors was obviously higher than that of the aneuploid (p=0.01), so he suggested that this technique is of a value in estimating the prognosis of retinoblastoma. The nuclear DNA content characterization was also carried out by means of DNA index and DNA histogram type assessments in a series of 21 retinoblastomas, 11 neuroblastoma, 1 gangleoneuroblastoma and 4 medulloblastoma by Dangou et al. [27], and they reported that retinoblastomas were significantly more aneuploid than neuroblastomas. The DNA histogram type showed that the high level of aneuploidy found in retinoblastoma corresponds to genotypically polymorphic tumors, and this could reflect serious degeneration of the genomic material in retinoblastoma.

In conclusion retinoblastoma samples showed high degree of p53 protein expression and high degree of aneuploidy which were related to the aggressiveness of histopathologic changes of retinoblastoma. Thus both p53 expression and DNA ploidy have been shown to be markers of aggressiveness of tumor behaviour in retinoblastoma and can help in the prediction of its prognosis.

References
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