S-Phase Fraction as a Predictor of Prognosis in Juvenile Respiratory Papillomatosis

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Objective: To determine whether DNA ploidy and the S-phase fraction are predictive of the clinical course in children with recurrent respiratory papillomatosis.

Design: Masked compression of DNA analysis findings to the clinical course of the disease.

Setting: Tertiary referral center.

Patients: All pediatric patients treated for recurrent respiratory papillomatosis at our institution between 1989 and 1995 who had adequate follow-up and whose primary biopsy specimen was available for examination. Fifty-five patients met these criteria.

Methods: Information was collected from the case notes on the patient’s age at presentation, sex, sites of disease, duration of active disease, and frequency of operative interventions. Flow cytometric analysis was performed on the archival paraffin-embedded primary biopsy specimen obtained at the initial surgical excision, providing DNA content and percentages of S-phase cells. The investigators who performed the DNA analysis were masked to the clinical course.

Results: The age of the patients at presentation ranged from 3 months to 16 years. Thirty patients had involvement in more than 1 anatomical site. The disease in 10 patients had spread to the distal tracheobronchial tree. The patients underwent a total of 1124 procedures, with a frequency range of 7 to 27 per year. All cell populations studied were diploid. The percentage of S-phase cells was significantly higher in the primary biopsy specimen from patients with disease characterized by more frequent recurrences, multiple sites, and distal extension (P < .05). In multiple regression analysis, the S-phase fraction was found to be an independent and powerful prognostic factor for aggressive disease.

Conclusions: The S-phase fraction may be predictive of the clinical course in patients with juvenile respiratory papillomatosis. Prospective studies are needed to assess the diagnostic and clinical value of our primary results and to determine whether DNA analysis can assist in identifying patients at increased risk for an aggressive clinical course.


RECURRENT respiratory papillomatosis (RRP) is the most common benign neoplasm of the larynx in children.1 Although histologically a benign neoplasm, RRP may be a serious and potentially life-threatening disease due to obstruction of the airway. Characteristically, the clinical course is unpredictable; some patients have a solitary lesion that responds to few treatments, while others have lesions that recur chronically, arising from multiple sites and spreading distally to the tracheobronchial tree, requiring multiple surgical procedures. One of the more perplexing issues regarding RRP that remains unresolved is how to predict the course and severity of disease in an individual patient. Despite attempts to link the clinical course and prognosis of RRP with the initial endoscopic findings,2 the histological findings,3,4 the human papillomavirus subtype,5 and viral coinfections,6 to our knowledge there is no way at present to identify those patients at risk for aggressive disease.

Analysis of nuclear DNA content has been used to evaluate malignant human neoplasms.8 Flow cytometry allows automatic quantification of the nuclear DNA content with cell-cycle analysis of a large number of individual cells.9 We used flow cytometry to analyze the DNA content in RRP biopsy specimens obtained at presentation. The objective of this study was to determine whether nuclear DNA analysis can assist in predicting the clinical course.
PATIENTS AND METHODS

PATIENTS

Eighty-eight patients in the pediatric age group (age at disease onset, <16 years) with the diagnosis of RRP were treated in our institution between 1989 and 1995. Patients who had adequate follow-up and in whom the paraffin blocks containing the formalin-fixed original (pretreatment) biopsy specimens could be obtained were included in the study. The charts of those patients were reviewed with respect to age at presentation, sex, sites of involvement (supraglottis, glottis, subglottis, trachea, bronchi, lungs, pharynx, and esophagus), number of recurrences, treatment modalities, and duration of clinically active disease. Clinical remission was defined as complete absence of gross papillomas in at least 2 consecutive examinations over a minimum of 12 months. Indication for surgery was hoarseness, airway obstruction, or detection of gross respiratory papilloma by flexible laryngoscopy. The average recurrence frequency for each patient was calculated based on the patient's total number of surgical procedures and the time of active disease. All patients had at least 18 months of follow-up.

FLOW CYTOMETRIC PROCEDURE

Flow cytometric DNA analysis was performed on paraffin sections of the biopsy specimen obtained at the time of the initial surgical excision. Reexamination of the histopathologic findings confirmed that all specimens contained respiratory papillomatosis. Final sectioning of the tissue for the extraction of nuclei was based on a histological review of the hematoxylin-eosin–stained slides to determine the adequacy of the papilloma mass and the density within the paraffin-embedded tissues. Three 40-µm sections from areas of high papilloma density in each paraffin block were then deparaffinized in xylene, rehydrated in graded alcohols, washed in water, and resuspended with 1.5 mL of 0.5% peptin at a pH of 1.5 in a 37°C water bath for at least 45 minutes, with vortexing at 5-minute intervals. The resulting nuclear yield was suspended in 3 mL of phosphate-buffered saline and filtered through a 30-µm nylon mesh. The suspension was then centrifuged, and the pellet was resuspended in a 50-µg/mL solution of propidium iodide (Sigma Chemical Co, St Louis, Mo). A single-cell suspension of normal lymphocytes activated by phytohemagglutinin P (Sigma Chemical Co) was used as the external positive control for DNA labeling. Chick red blood cells with a DNA content of 0.35% of normal human diploid cells were used as the internal positive control for DNA labeling. Nuclear DNA content was measured with a flow cytometer (EPICS C, Becton Dickinson, Sunnyvale, Calif), equipped with a 5-W argon laser run at a wavelength of 514 nm. Fluorescence was collected by a bandpass filter at 488 nm. The percentages of the total cell population in S-phase on the red fluorescence histogram were calculated by multiple-option cell-cycle fitting (Multicycle, Phoenix Flow System, San Diego, Calif). The DNA index was defined as the ratio of the G0/G1 DNA content of sample cells to that of the phytohemagglutinin-activated normal G0/G1 lymphocytes. Diploidy was defined by a DNA index of 1.00 and aneuploidy by a DNA index of more than 1.10 or less than 0.90. The investigators analyzed all DNA histograms without knowledge of the patient's clinical course.

STATISTICAL ANALYSIS

Statistical analysis was performed in our university computer center using a statistical software package (SAS Inc, Cary, NC). The Kendall nonparametric correlation coefficient was used to calculate associations among the variables. The prognostic significance of the S-phase fraction was evaluated by multiple Poisson regression analysis. \( P < .05 \) was considered significant.

RESULTS

The original biopsy specimen, obtained at the time of the initial diagnosis, was available for examination in 55 cases. The patients' age at onset of disease ranged from 3 months to 16 years, and the male-female ratio was 1.3:1. Twenty-five patients had involvement in a single site, most commonly the glottis, and 30 patients had involvement in multiple sites. Ten patients had distal spread to the tracheobronchial tree, and 11 patients had extralaryngeal (pharynx and/or esophagus) disease. The patients underwent a total of 1124 procedures, and the frequency of operations per year ranged from 7 to 27 (mean ± SE, 5.7 ± 0.77). Follow-up ranged from 18 months to 6 years. Fourteen patients entered complete clinical remission during the study period.

FLOW CYTOMETRIC FINDINGS

Flow cytofluorometric analysis of the initial biopsy specimen was performed in 55 cases. The cellularity of samples was sufficiently rich to allow analysis of all samples. Multiple specimens from each papilloma were analyzed, and no case of discordant DNA analysis results was noted. The coefficient of variation averaged 5.5 (range, 2.8-7.0). In all samples \( (n = 55) \), the DNA index value was between 0.90 and 1.10, indicating the diploidy of the cells studied.

The S-phase fraction could be determined for all of the 55 papilloma cell populations identified in the DNA histograms. The percentage of cells in the S phase ranged from 0.7 to 8.0 (mean ± SE, 3.3 ± 0.34).

CLINICOPATHOLOGIC CORRELATIONS

No correlation was found between the S-phase fraction and the age at onset of disease, sex, site of biopsy, or clinical remission.

The S-phase fraction correlated significantly with the frequency of surgical procedures \( (P < .001) \) (Figure 1), the number of anatomical sites that were involved by the disease \( (P < .001) \) (Figure 2), and distal spread to the tracheobronchial tree \( (P < .05) \) (Figure 3). In a multiple Poisson regression analysis, the S-phase fraction was found to be an independent and powerful predictor of...
The frequency of surgical procedures (P<.001), sites of involvement (P<.05), and distal spread (P<.05).

**COMMENT**

The underlying mechanisms for the abnormal proliferation of respiratory epithelial cells leading to the formation of papillomas is unclear. Morphological changes, such as squamous metaplasia, abnormal squamous keratinization, multinucleation, atypical parabasal cells, basal hyperplasia, and increased mitoses, are frequently observed.3,7 These findings suggest modification of normal epithelial differentiation and proliferation. Demeter et al10 demonstrated an induction of proliferating cell nuclear antigen in cells of human papillomavirus–infected lesions. They postulated that proliferating cell nuclear antigen, along with other host replication genes, is induced by viral gene products to remobilize the cellular DNA replication machinery so that vegetative viral replication can take place in differentiated cells that otherwise do not support DNA replication. It has been suggested that human papillomavirus may deregulate the cell cycle and increase epithelial cell production by regeneration and/or degradation of tumor suppressor gene products, thus preventing them from carrying out their function as regulators of cell growth.11,12

Several methods can be used to characterize proliferating cells. Flow cytometric analysis of nuclear DNA content is being used to evaluate proliferating cells in malignant tumors.13,14 However, this method is rarely used to analyze cell proliferation in benign lesions. Flow cytometry was found to be an effective method with which to evaluate nuclear DNA in RRP cells. Using this method, we were able to determine DNA ploidy and the distribution of the cells in the cell cycle in papilloma specimens. All the specimens in our study had diploid DNA content. This finding is consistent with the findings of chromosomal analysis of recurrent laryngeal papillomas, which revealed that all cells analyzed had normal karyotypes.15

The S-phase fraction was significantly higher in the initial biopsy specimen from patients who had an aggressive clinical course that was characterized by multiple anatomical sites, extensive spread of disease, and higher frequency of recurrence. However, the patients described herein were drawn from a group of 88 patients with RRP who were under our care during the study period. Moreover, because our institution is a referral tertiary center, the proportion of patients with more severe disease who were included in this study may be higher than in the general pediatric RRP population. Therefore, the observed distribution of the S-phase fraction in this study may not be representative of all pediatric patients with RRP.

Our primary results suggest that cell proliferation can be increased in more aggressive RRP and may participate in the pathogenesis of these lesions. DNA analysis during different clinical stages of the disease and other methods for evaluating cell proliferation, as well as further prospective studies, are needed to assess the diagnostic and clinical value of our primary results. A clarification as to the role of cell proliferation in establishing the severity of RRP will improve our knowledge of the pathogenesis of the disease and may provide a prognos-
tic indicator as to the course and management of juvenile respiratory papillomatosis.

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REFERENCES