Expressions of proliferation markers (Ki-67, proliferating cell nuclear antigen, and silver-staining nucleolar organizer regions) and of p53 tumor protein in gestational trophoblastic disease

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OBJECTIVE: This study was undertaken to determine whether the expressions of 3 proliferation markers (Ki-67, proliferating cell nuclear antigen, and silver-staining nucleolar organizer regions) and of p53 tumor protein could differentiate spontaneous abortions from gestational trophoblastic diseases and also discriminate among gestational trophoblastic disease subgroups.

STUDY DESIGN: Twenty-two partial hydatidiform moles, 17 complete hydatidiform moles, 6 invasive hydatidiform moles, and 20 nonhydropic spontaneous abortions (control group) were evaluated by means of immunohistochemical techniques with antibodies to Ki-67, proliferating cell nuclear antigen, and p53. One-step silver staining was used to detect silver-staining nucleolar organizer regions.

RESULTS: The expressions of Ki-67, proliferating cell nuclear antigen, silver-staining nucleolar organizer regions, and p53 were significantly higher in the gestational trophoblastic disease group than in the control group. The results of linear discriminant analysis showed that silver-staining nucleolar organizer region count had the highest sensitivity and specificity (93.3% and 100%, respectively) for distinguishing gestational trophoblastic disease from spontaneous abortion. Sensitivity and specificity for discriminating gestational trophoblastic disease from spontaneous abortion increased to 100% when all four markers were used together. Proliferating cell nuclear antigen was found to be the best discriminating variable for differentiating among gestational trophoblastic disease subgroups.

CONCLUSION: Our findings suggest that expressions of Ki-67, proliferating cell nuclear antigen, silver-staining nucleolar organizer regions, and p53 may aid in the diagnosis of gestational trophoblastic diseases. These fairly rapid, simple, and economic techniques could serve as a useful adjunct to conventional methods in the diagnosis of gestational trophoblastic diseases. (Am J Obstet Gynecol 2001;184:567-74.)

Key words: Gestational trophoblastic disease, hydatidiform mole, immunohistochemical analysis, Ki-67, p53, proliferating cell nuclear antigen, silver-staining nucleolar organizer regions

Gestational trophoblastic diseases belong to a group of entities characterized by abnormal proliferation of the trophoblast and are classified as partial hydatidiform moles, complete hydatidiform moles, invasive hydatidiform moles, choriocarcinomas, and placental-site trophoblastic tumors. The differential diagnosis of a molar gestation versus a nonmolar abortion should be stated clearly, because women with molar pregnancies are at increased risk for the subsequent development of malignant sequelae and persistent gestational trophoblastic disease.1,2

Despite well-described histopathologic criteria, the distinction of spontaneous abortion from partial hydatidi-
organizer regions (AgNORs), have been established as a valuable reflection of the tissue proliferative compartment and thus could be of value in studying the biologic behavior of molar gestations. On the other hand, the nuclear phosphoprotein p53, the product of the TP53 tumor suppressor gene, is present in extremely small amounts in normal tissues and is almost undetectable in conventional immunostaining assays because of its short half-life; however, mutations in TP53 are the most frequently described genetic changes in a wide variety of human tumors and can be detected by immunohistochemical methods. We therefore also decided to investigate the overexpression of p53 protein in spontaneous abortions versus cases of gestational trophoblastic disease.

Immunohistochemical methods for detection of Ki-67, PCNA, and p53 are a relatively simple alternative to the more complex techniques. One of the advantages of these methods is the ability to apply them retrospectively to sections of routinely formalin-fixed and paraffin-embedded tissue. Another advantage is that there is no need for expensive or sophisticated equipment, unlike with deoxyribonucleic acid ploidy analysis techniques.

Current data concerning the importance of cell proliferation markers suggest that both Ki-67 and AgNORs could be used in the differential diagnosis of gestational trophoblastic disease, whereas PCNA seems to have limited value. Although the studies related to p53 protein have shown promising results, it is not clear why p53 expression is higher in gestational trophoblastic disease than in normal placentas.

The aim of this study therefore was to evaluate the expressions of 3 proliferation markers (Ki-67, PCNA, and AgNORs) and of p53 tumor protein in gestational trophoblastic disease and also to assess the values of these markers both individually and as a group in the differential diagnosis of gestational trophoblastic disease subgroups and spontaneous abortions.

### Material and methods

#### Patients

Twenty-two partial hydatidiform moles, 17 complete hydatidiform moles, 6 invasive hydatidiform moles, and 20 first-trimester nonhydropic spontaneous abortions (control group) diagnosed previously in the Department of Pathology, School of Medicine, Ankara University, Ankara, Turkey, were included in the study after reevaluation of each case to confirm the diagnosis. The histologic features of the specimens were assessed according to the diagnostic criteria of Szulman and Surti.5, 27

The median ages of the patients were 23 years (range, 18-38 years) in the partial hydatidiform mole group, 25 years (range, 18-42 years) in the complete hydatidiform mole group, 32 years (range, 21-50 years) in the invasive hydatidiform mole group, and 27 years (range, 19-43 years) in the spontaneous abortion group. Patient data included gestational age, gravidity, parity, abortions, and pre-evacuation and postevacuation serum β-human chorionic gonadotropin levels.

#### Immunohistochemical studies

Multiple 6-µm-thick sections of representative formalin-fixed, paraffin-embedded tissues were cut for immunohistochemical studies. A streptavidin-biotin-peroxidase technique (Zymed Laboratories Inc, South San Francisco, Calif) was used for the detection of Ki-67 (prediluted MIB1; Ylem Srl, Avezzano, Italy), PCNA (prediluted PC10; Ylem), and p53 (1:50;
DO7; Zymed Laboratories) with the antigen retrieval microwave method (BioGenex Laboratories, San Ramon, Calif).

AgNOR solution was prepared by mixing 2 g/dL gelatin in 1 mL/dL aqueous formic acid with 50 g/dL aqueous silver nitrate in a ratio of 1 to 2. The slides were incubated for 30 minutes at room temperature in the dark and then washed under tap water.

All immunostained sections were examined by the same two observers with a ×400 objective under the light microscope (Olympus Bx50; Olympus Optical Co, Ltd, Tokyo, Japan) for evaluation of Ki-67, PCNA, and p53 expressions. In the abortion specimens and hydatidiform moles 1000 villous cytotrophoblastic cells were counted in each case for Ki-67 and PCNA expression. All stained nuclei were scored as positive, regardless of staining intensity. The nuclear labeling index values for MIB1 (Ki-67) and PC10 (PCNA) immunoreactivity were determined by scoring positive nuclei per total number of counted nuclei for 1000 cytotrophoblastic cells in each case.

The p53 expression was determined by evaluating the percentage positivity observed in the cytotrophoblastic cell population within placental tissue. AgNORs in 100 villous cytotrophoblastic cells for each case were counted under a ×100 oil-immersion lens. A nucleolar cluster was counted as a single AgNOR, irrespective of the number of dots within the nucleolus.

Tissue sections of prostatic carcinoma served as positive control preparations for all antibodies studied.

**Statistical analysis.** The results obtained from the case groups and the control subjects were compared for all the parameters included in the study (age, gestational age, serum β-human chorionic gonadotropin values, and Ki-67, PCNA, p53, and AgNOR expressions) by means of the Student t test, χ2 analysis, and the Mann-Whitney U test. The control group and case groups were analyzed by means of linear discriminant analysis with all the parameters included in the study to assess the discriminatory value of each parameter. The results were expressed as mean ± SD. Statistical significance was determined at P < .05 on the basis of 2-tailed tests.

**Results**

Immunostaining for Ki-67, PCNA, AgNOR, and p53 was almost entirely limited to cytotrophoblastic cell nuclei, with a granular pattern in every case. Ki-67 and PCNA index values were significantly higher in the case group than in the control group (Table I). Invasive hydatidiform mole showed the highest Ki-67 and PCNA expressions among the case subgroups, followed by complete hydatidiform mole and partial hydatidiform mole (Table II). There was also a statistically significant difference between the spontaneous abortion group and the gestational trophoblastic disease groups for all the parameters studied (P < .001). Of those four parameters, only Ki-67 and PCNA showed the most significant difference among gestational trophoblastic disease subgroups (P < .001; Fig 1, A-D, and Figs 2 and 3).

The expression of p53 was significantly higher in molar placentas than in nonmolar placentas (P < .001; Table I). A significant difference in p53 expression was also observed between partial hydatidiform mole and complete hydatidiform mole (P < .05), whereas no such difference was detected between complete hydatidiform mole and invasive hydatidiform mole (P > .05) (Fig 1, E and F, and Fig 4).

The findings in each of the diagnostic categories for AgNOR staining are shown in Tables I and II. There was a statistically significant difference between the spontaneous abortion group and the gestational trophoblastic disease groups (P < .001). Among the molar placentas, AgNOR counts in partial hydatidiform moles were higher than in complete hydatidiform moles (P < .001) and in invasive hydatidiform moles (P < .001; Fig 1, G and H, and Fig 5).

The results of linear discriminant analysis showed that AgNOR expression had the highest sensitivity and specificity (93.3% and 100%, respectively) for distinguishing gestational trophoblastic disease from the spontaneous abortion group (Table III). The sensitivity and specificity values increased to 100% when all the variables were used together in the stepwise discriminant analysis.

On the other hand, PCNA was found to be the best variable for discriminating among gestational trophoblastic disease subgroups. The results showed that 100% of patients with partial hydatidiform mole, 94.1% of patients with complete hydatidiform mole, and 83.3% of patients with invasive hydatidiform mole had correct diagnoses made by means of PCNA expression. The results of each variable for discrimination of gestational trophoblastic disease subgroups are presented in Table IV. There was no improvement in the percentage of cases diagnosed in each gestational trophoblastic disease subgroup when all the variables were included in the discriminant analysis (Table IV).

**Comment**

This immunohistochemical study was designed to determine whether proliferation markers and p53 protein overexpression in trophoblastic tissue would have any discrimi-

| Table III. Sensitivities and specificities of each parameter used in discriminant analysis and of all parameters combined |
|-----------------|-----------------|-----------------|
| Sensitivity (%) | Specificity (%) |
| Ki-67 | 84.4 | 100 |
| PCNA | 86.7 | 100 |
| AgNOR | 95.3 | 100 |
| p53 | 64.4 | 100 |
| All variables | 100 | 100 |
Fig 1. Immunohistochemical staining with Ki-67 antibody in partial (A) and complete (B) hydatidiform moles, with PCNA antibody in spontaneous abortions (C) and complete hydatidiform moles (D), with p53 antibody in spontaneous abortions (E) and invasive hydatidiform moles (F), and with AgNORs in partial (G) and complete (H) hydatidiform moles. Positive and negative nuclear stains are indicated by thick arrows and thin arrows, respectively. (A and B: Original magnification ×100. C to F: Original magnification ×200. G: Original magnification ×100. H: Original magnification ×400.)
natory value for spontaneous abortion and gestational trophoblastic disease. To the best of our knowledge this is the first study to investigate proliferative activity extensively by measuring the expressions of 3 proliferation markers together with p53 protein for the diagnosis of spontaneous abortions and gestational trophoblastic disease.

In spontaneous abortions the mean counts of cytotrophoblastic cells staining positively for Ki-67, PCNA, AgNOR, and p53 were much lower than those in gestational trophoblastic disease. This significant difference in the immunoreactivity of markers between spontaneous abortions and gestational trophoblastic disease is probably caused by the aberrant trophoblastic proliferation pattern characteristic of hydatidiform moles.

The highest expressions of Ki-67 and PCNA were observed in the invasive hydatidiform mole group, which suggests a more pronounced trophoblastic hyperplasia and proliferative activity in invasive moles. Although in...
In this study the most significant overexpression of p53 was observed in the invasive hydatidiform mole group, consistent with the results of other previous studies,

24 the discriminatory value of p53 was lower than that of PCNA, which yielded the best diagnostic value for gestational trophoblastic disease subgroups. Because no improvement was achieved after including all variables in the analysis, PCNA proved to be the sole discriminant necessary for gestational trophoblastic disease subgrouping.

Molykutty et al26 reported that PCNA immunoreactivity in first-trimester molar placentas was significantly higher than that in normal placentas. They also noted that the correlation of the staining score with the regression pattern of the tumor was significantly increased in the chemotherapy group relative to the spontaneously regressing group.

However, a number of studies in the literature have failed to find any important differences among the PCNA counts of gestational trophoblastic disease subgroups.15, 17, 18, 23 Schammel and Bocklage15 noted a significant difference in Ki-67 immunoreactivity but not in PCNA or p53 expression between molar and nonmolar pregnancy and between complete hydatidiform mole and partial hydatidiform mole. Suresh et al,9 in a study of PCNA immunoreactivity in gestational trophoblastic disease, reported that there was a significant difference between the PCNA counts in normal first-trimester placentas and both hydropic abortions and partial hydatidiform moles, whereas the difference in PCNA counts between hydropic abortions and partial hydatidiform moles was not significant. In their analysis PCNA immunoreactivity of villous cytotrophoblastic cell population in partial hydatidiform moles was significantly lower than that in normal first-trimester placentas. They noted that a distinguishing feature in the diagnosis of partial hydatidiform mole was the atypical pattern of trophoblastic proliferation, with a focal, multifocal, or circumferential pattern rather than the polar or lateral growth seen in the normal first-trimester placenta.

In another study23 molar placentas showed moderate to strong PCNA immunoreactivity, whereas cytotrophoblastic cells in normal placental tissue exhibited insignificant staining. Contrary to our results, the findings of the study demonstrated no significant quantitative difference between partial and complete hydatidiform moles with respect to PCNA expression.

On the other hand, in this study the extent of PCNA immunoreactivity was minimal in normal placentas and more extensive in hydatidiform moles, particularly in invasive hydatidiform moles. The more significant PCNA overexpression in invasive hydatidiform mole than in complete and partial moles is in keeping with the more pronounced trophoblastic hyperplasia and proliferative activity of invasive hydatidiform moles.

The high degree of variability in PCNA expressions reported in the literature may be explained by different technical factors in the microwave antigen retrieval methods, such as storage, extent of fixation, or staining. Furthermore, different areas of the same tissue may manifest heterogeneity with respect to the proliferation of cytotrophoblasts. As with any proliferation index, there are likely to be regional variations within a tissue section. In addition, this study was designed on the basis of morphologic criteria of Szulman and Surti.5, 27 Reliance on a given standard for morphologic classification may influence the results.

The p53 immunoreactivity showed the lowest sensitivity for distinguishing spontaneous abortion from gesta-
tional trophoblastic disease in this study. Similarly, the diagnostic value of p53 was lower than those of Ki-67 and PCNA when used for gestational trophoblastic disease subtyping. These results suggest that p53 expression may be an insufficient marker for diagnosis of gestational trophoblastic disease and differentiating gestational trophoblastic disease subgroups relative to the other markers studied. The increased overexpression of p53 in gestational trophoblastic disease seems to correlate with higher trophoblastic proliferation rate found mainly in complete and invasive hydatidiform moles. Also, the staining pattern was intense and extensive, involving mainly the cytotrophoblastic cells in the invasive hydatidiform mole group. That the p53 immunoreactivity was seen in primarily the germinative cell layer supports the contention that p53 expression could be an indicator of proliferative activity. That the relationship between gestational trophoblastic disease and p53 positivity was weaker than that of other markers could lead to the conclusion that the expression of p53 protein in this disease may not be very significant. The p53 overexpression, as indicated by immunohistochemical staining, could be the result of up-regulation of the TP53 tumor suppressor gene providing a mechanism for controlling trophoblastic proliferation and thus perhaps transformation and malignancy. However, the possible role of p53 in the pathogenesis of gestational trophoblastic disease needs to be studied.

Cheung et al\textsuperscript{20} reported a striking difference in the levels of p53 ribonucleic acid between normal placentas and complete hydatidiform moles at similar gestational ages and suggested that p53 overexpression in hydatidiform moles could modulate excessive trophoblastic proliferation. In an immunohistochemical study Persaud et al\textsuperscript{25} recorded that the mutation of the TP53 tumor suppressor gene, as demonstrated by the overaccumulation of p53 protein, was seen primarily in choriocarcinomas and nonvillous trophoblastic elements of hydatidiform moles. However, they also found that p53 overaccumulation was uncommon in partial hydatidiform moles and was minimal or absent in normal placentas.

In this study the sensitivity and specificity increased when all 3 proliferation markers and p53 results were included in the discriminant analysis. Although 100% sensitivity and specificity were reached when all 3 proliferative markers and p53 results were included in the study, the sensitivity and specificity of AgNOR counts alone were sufficiently high to be used in the differential diagnosis of molar versus nonmolar placentas. On the other hand, invasive hydatidiform mole, which shows the greatest degree of trophoblastic proliferation among the 3 subgroups of gestational trophoblastic disease, had moderately increased AgNOR counts, in contrast to the highest AgNOR immunoreactivity found in the partial hydatidiform mole group. Thus AgNOR counts in villous cytotrophoblastic cells did not seem to correlate with proliferative activity, as demonstrated by Ki-67 and PCNA. If it is considered that placentas from partial hydatidiform moles are generally triploid, our findings might suggest that the number of AgNORs may reflect hyperploidy rather than proliferative activity in the partial hydatidiform mole group, as has been postulated in previous reports.\textsuperscript{14,19} On the other hand, a strong positive correlation has been noted between deoxyribonucleic acid cytometric data or proliferation markers (Ki-67) and AgNOR content of the cells. Tumors with a high proportion of cells in S phase or increased Ki-67 expression tend to have higher numbers of AgNORs.\textsuperscript{11} In this study the relatively poor results of AgNOR compared with other variables for diagnosis of gestational trophoblastic disease subgroups seem to be rather discouraging for future studies. The significance of AgNOR expression in gestational trophoblastic disease needs further evaluation because of the conflicting reports in the literature.\textsuperscript{11, 14, 19, 25}

The results of this study therefore show that the evaluation of expressions of proliferation markers Ki-67, PCNA, and AgNOR and of the expression of p53 tumor protein in the cytotrophoblastic cells contributes to a reliable discrimination between spontaneous abortions and gestational trophoblastic disease. It further discriminates gestational trophoblastic disease subgroups. These fairly simple, rapid, and inexpensive immunohistochemical techniques could serve as useful adjuncts to conventional methods of diagnosis in suspected gestational trophoblastic disease. Further prospective studies could also help to determine the prognostic values in gestational trophoblastic disease of the expressions of these proliferation markers and of p53 tumor protein.
REFERENCES


