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ORIGINAL RESEARCH

Discovery of the prognostic marker in the whole blood sample of gestational trophoblastic neoplasia beyond immunohistochemical tissue markers: *mdm2*

Burcu Ozbakir[1,*], Aytekin Altintas[2]

[1]Department of Obstetrics & Gynaecology, Faculty of Medicine, Near East University, 99138 Nicosia, Turkey
[2]Department of Obstetrics & Gynaecology, Faculty of Medicine, Çukorova University, 01330 Sarıcam, Turkey

***Correspondence**
drburcuburcu@hotmail.com;
burcu.ozbakir@med.neu.edu.tr
(Burcu Ozbakir)

Abstract

Gestational trophoblastic disease (GTD) is an abnormal trophoblastic proliferation disease that may show malignant progression. Despite reports on the clinical and laboratory parameters for the projection of GTD, there is still a lack of histopathologic or genetic prognostic markers for patients with higher risks of malignant progression and high-risk gestational trophoblastic neoplasia. The primary aim of this study was to identify a marker other than tissue markers for evaluating the progression potential of GTD to gestational trophoblastic neoplasia (GTN). The secondary aim was to determine the tissue marker capacity to detect the progression to neoplasia. The study design was a case-control. The data of 81 patients diagnosed with GTD and 23 control were assessed. Their detailed obstetric and gynecological history were recorded, and their whole blood sample was obtained for genetic evaluation of the *mouse double minute 2* (*mdm2*) gene expression. We also evaluated the curettage specimens for p53, *c-erythroblastic oncogene B-2* (*c-erbB-2*), and ki67 protein expression. The expression of p53 expression was significantly increased in GTD patients and significantly higher in the GTN progressing group than in the spontaneous remission group. Although blood *mdm2* gene was significantly different between the low and high-risk GTN subgroups, its expression was not different between the GTD or GTN groups. Additionally, a significant increase in c-erbB-2 was observed in the GTN group. Mdm2 might be a promising blood prognostic factor throughout the course of GTD. p53 and c-erbB-2 might be used as predictive indicators for the early diagnosis of GTN progression. Altogether, these markers may help identify patients with high risks of malignant progression, guide earlier aggressive treatment and increase treatment outcomes.

Keywords

mdm2; p53; c-erbB-2; Ki67; Gestational trophoblastic disease; Gestational trophoblastic neoplasia

1. Introduction

Gestational trophoblastic disease (GTD) comprises a wide range of conditions defined by abnormal trophoblastic proliferation [1]. The disease is histologically classified into a hydatidiform mole, which can be subdivided into complete and partial subgroups, invasive mole, choriocarcinoma, placental site trophoblastic tumor, and epithelioid trophoblastic tumor [2]. Except for hydatidiform mole, the other types of GTD can develop into an invasive or metastatic disease called gestational trophoblastic neoplasia (GTN) [3]. The malignancy potential, invasion, and metastasis make GTD an important condition for evaluating the prognostic factors to improve the outcomes of the disease. The World Health Organization (WHO) staging and combined scoring system of International Federation of Gynecology and Obstetrics (FIGO) Oncology Committee is currently used for its prognostic evaluation, in which FIGO stage 1–3 and WHO score <7 are considered low-risk GTN, and FIGO stage 4 and WHO score ≥ 7 are considered high-risk GTN [4]. Even though there are some clinical and laboratory-based parameters for projecting the prognosis of the disease, there still is a lack of histopathologic or genetic predictors. In addition, prognostic markers are urgently needed to identify patients at high risk of disease progression, provide timely treatment and potentially suppress its malignant transformation.

p53 is a well-known tumor suppressor gene. The p53 protein controls cell cycle, apoptosis, and DNA repair based on the extent of cellular damage [5]. Cell division is interrupted at the G1-S phase when the damage is small, whereas apoptosis is triggered in bigger damage to suppress malignant cellular transformation [6, 7]. p53 also controls trophoblastic cellular differentiation via target gene expressions [8].

The *mdm2* gene is a proto-oncogene suppressing p53 [5].

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Mutation of the *p53* gene in both alleles may lead to its dysfunction and carcinogenesis while a mutation on the *mdm2* gene affecting the *p53* gene may lead to uncontrolled p53 suppression and carcinogenesis [5, 9].

c-erbB-2 is also a proto-oncogene expressed in embryogenesis, especially in cellular proliferation and differentiation physiology [10].The pathological overexpression of this gene may result in oncogenesis [11].

The Ki67 gene's protein product is a cell proliferation marker expressed during the active phases of cellular division (G1, S, and G2). It is used to evaluate the cell activity and growth fraction of normal or neoplastic tissues providing an indicator of patients' prognosis [12].

This present study aimed to evaluate the prognostic value of p53, c-erbB-2, and Ki67 in curettage samples and assess the association of *mdm2* gene expression in the blood sample with the tendency of neoplastic progression.

2. Methods

2.1 Population

In this retrospective case-control study, we evaluated 81 GTD cases admitted to the Obstetrics & Gynaecology Department of the Gynaecological Oncology Unit of the Cukurova University Medical Faculty in the past 12.5 years. The control group was collected in the same period as the case group and from the patients admitted to our department with a first-trimester miscarriage or needed medical termination with no diagnosis of GTD, the control group consisted of 23 patients. Of the initially 104 cases and control patients, 102 of them were reached and the detailed history was taken from the patients; as 2 of the patients were deceased, the detailed history of the patients was recorded from one of the first-degree relatives. The relevant clinical data collected included the age at diagnosis, β human chorionic gonadotropin (β-hCG) level at diagnosis, previous GTD history, pregnancy history (gravidity, parity, abortion), the interval between the last pregnancy and GTD, and previous infertility and/or infertility treatment history (Table 1).

The cases were grouped into four main categories: (1) complete hydatidiform mole with complete remission; (2) partial hydatidiform mole with complete remission; (3) low-risk GTN; (4) high-risk GTN. The WHO scoring and combined FIGO staging system were used to differentiate between low-risk and high-risk GTN. There was no invasive mole case in our study.

From the 104 patients including the cases and the controls, we also assessed the curettage specimens of 79 patients at the time of the diagnosis at the Pathology Department of the Cukurova University Medical Faculty, and the whole blood sample of 71 patients was collected. The 25 patients did not have their diagnosis in our hospital, and we could not reach the pathological samples. 2 of the patients were deceased that's why blood samples could not be provided and from the alive 102 patients in total 31 patients were not suitable to provide a blood sample for our research. The grouping of the patients is summarized in Table 2.

2.2 Immunohistochemistry

Briefly, 5 μm thick sections from formalin-fixed and paraffin-embedded tissue blocks were obtained. They were incubated for 30–45 minutes at 60 °C, deparaffinized in three xylene chalets and three alcohol chalets three times for five minutes each, then rehydrated. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution for five minutes, and the sections were rewashed.

The consecutively taken serial slides were incubated in a solution buffered with Tris Ethylenediaminetetraacetic acid (EDTA) at pH 9 for p53 and Tris EDTA at pH 8 for c-erbB-2 and Ki67 in a microwave oven (800 W) twice for seven minutes each. They were left to cool at room temperature for 40–45 minutes, then washed with 0.01 M phosphate-buffered saline (PBS) (pH 7.2–7.4). The slides were treated with primary antibodies (1/35 diluted Novocastra™ Liquid Mouse Monoclonal Antibody (NCL)-p53-D07 Novocastra Monoclonal Mouse Anti-human for p53, 1/45 diluted NCL-CB11 Novocastra Liquid Mouse Monoclonal Antibody for c-erbB-2 and 1/50 diluted NCL-L-Ki67-MM-1 Novocastra Liquid Mouse Monoclonal Antibody for Ki67) for 90 minutes at +4 °C. They were then washed in PBS for 3–5 minutes. The sections were incubated with biotin (DAKO-Labeled Streptavidin-Biotin (LSAB) + System-horseradish peroxidase (HRP)) for 20 minutes, then with avidin (DAKO-LSAB + System-HRP) for 30 minutes at room temperature. The slides were rewashed in PBS for 3–5 minutes. Aminoethyl carbazole (AEC) chromogen was used for chromogenic reactions. Then, the sections were washed, counterstained with Mayer Haematoxylin, and mounted with DAKO Faramount Aqueous Mounting. Appropriate positive and negative controls were used for each case. The protein expression in each specimen was evaluated by a single pathologist from the same focus of the tissue biopsy under a light microscope. Two slides per case (n = 280 slides total) were evaluated for immunohistochemical analysis.

The rate of p53 expression was reported as the percentage of positive nuclear immunoreactivity within approximately 1000 cytotrophoblasts. To semi-quantitatively grade, p53 staining intensity, (–) was used to represent 0–4% of cell staining, (+) for 5–25% of cells stained, (+2) for 26–50% of cells stained, (+3) for 51–75% of cells stained, and (+4) for $>$75% of cells stained. We also evaluated the mutant type p53, which was characterized by one of the three patterns: diffuse positive $>$75% of the cells, complete negative staining, or cytoplasmic reaction only.

To score c-erbB-2 staining intensity, we evaluated approximately 1000 cytotrophoblasts and syncytiotrophoblasts' membranous staining percentage and intensity. (–) was denoted when none of the cell membranes were stained, (+1) for faint membranous staining in a few cells, (+2) for weak to moderate complete membranous staining in approximately half of the cells, and (+3) for strong for complete membranous staining in most/all of the cells.

Ki67 expression rate was reported as the percentage of positive nuclear immunoreactivity within approximately 1000 villous cytotrophoblasts.

TABLE 1. Demographic features of the case and control groups.

Variables	Case Group	Control Group	*p*-value
Age	32.50 ± 10.79	31.00 ± 5.97	>0.05
Gravidity	3.60 ± 3.36	1.87 ± 1.45	>0.05
Parity	2.70 ± 2.98	1.13 ± 1.01	0.05
Abortion	0.90 ± 1.19	0.74 ± 0.86	>0.05
Infertility history	7.40%	4.30%	>0.05

TABLE 2. Distribution of the patient groups.

Variables	No. of cases	Cases with curettage sample	Cases with blood sample
Complete mole with remission	29	23	21
Partial mole with remission	10	7	5
Low-risk GTN	18	14	8
High-risk GTN	24	14	19
Control	23	21	18
Total	104	79	71

GTN: gestational trophoblastic neoplasia.

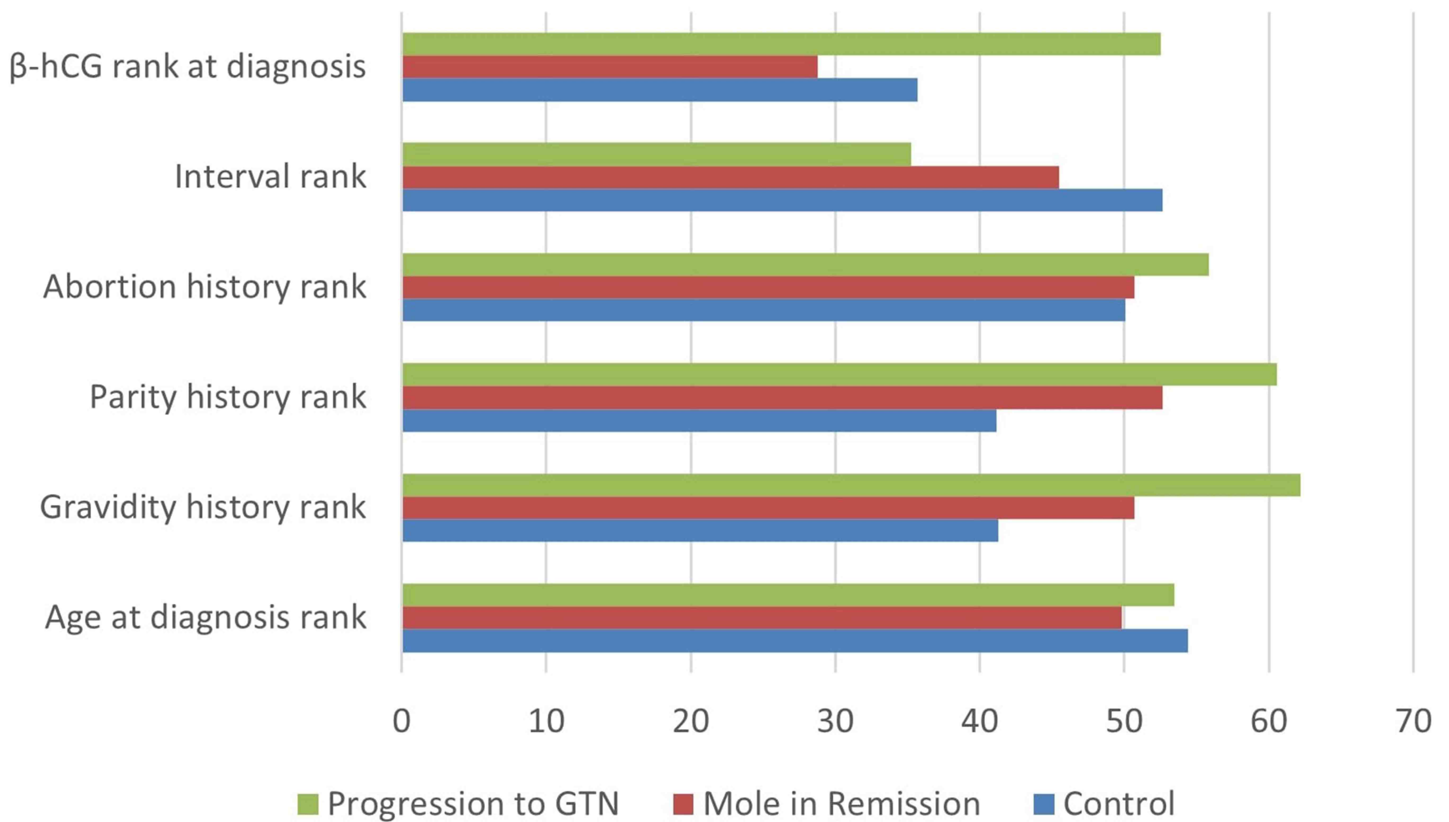

FIGURE 1. Evaluation of the clinical parameters between the mole hydatidiform with complete remission, the GTN group, and the control group. hCG: human chorionic gonadotropin.

2.3 Genetic studies

Briefly, 9 mL of whole blood was collected in three tubes with EDTA (3 mL each). One of the tubes was used to isolate DNA with a DNA isolation kit (Lot number: 04379012001, MagNA Pure LC DNA Isolation Kit, Roche Diagnostic, Mannheim, Germany) using the robotic method and was kept at −20 °C. Another tube was centrifuged at 2500 rpm for 10 minutes and the plasma was collected. One tube was tested for whole blood count and the leucocyte count was stabilized to 3 million leucocytes per patient. The Erythrocyte Lysing Solution was used to clear erythrocytes from the area. mRNA isolation was conducted using the mRNA isolation kit (Lot number: 04686918001, MagNA Pure LC mRNA HS Isolation Kit, Roche Diagnostic, Mannheim, Germany) with the robotic method and kept at −20 °C.

Tissues embedded in paraffin were deparaffinized using the xylene solution, ethanol, and distilled water. The tissues were kept in an Overnight Lyse was treated with a MagNA Pure LC mRNA II Tissue Isolation Kit using the robotic method. Tissue mRNA was isolated and kept at −20 °C.

Isolated mRNA products were transformed to cDNA with the Transcriptor cDNA synthesis Roche Kit. In total, 20 μL was added to each well as the last volume, treated at 25 °C for 10 minutes, 50 °C for 60 minutes and 85 °C for 5 minutes for Transcriptor Reverse Transcriptase inactivation, then placed in cold blocks. The isolated mRNAs for gene expression were assessed with the Universal Probe Library (UPL) ready probe and primer designs in LC 480 II machine for Real-Time Polymerase Chain Reaction (PCR) purposes. Mdm2 expression and reference housekeeping gene beta-actin gene expressions with suitable House Keeping Gene set (ACTB) and ROCHE UPL Probe House Keeping Gene set for each sample. Expression results were obtained using the Relative Quantification software of the LC480 II Real-Time PCR machine. The *mdm2*/reference gene expression was calculated for each sample.

2.4 Statistics

Statistical analyses were performed using the Statistical Package for Social Science software (PC Windows version 17, IBM, New York, NY, United States). Nominal data were analyzed using the Chi-Square Test, whereas non-nominal data with the Mann-Whitney test to compare medians between two groups. Variance analysis was performed to analyze differences among different groups. Correlation between numerical data was determined using the Pearson correlation test. A *p*-value < 0.05 was considered statistically significant.

3. Results

Demographic characteristics such as age at diagnosis, pregnancy history (gravidity, parity, abortion), and infertility history were similar between the case and control groups. As there was only one case with GTD history and the sample size was within the limitations, the GTD history variable was excluded from the research. The interval between previous pregnancy and GTD was 35.2 $\pm$ 31.65 months in mean.

The demographic characteristics were evaluated between the control group and the complete hydatidiform mole with complete remission, partial hydatidiform mole with complete remission, low-risk GTN, and high-risk GTN. Significant results were observed only for the interval between the previous pregnancy ($p = 0.029$). The highest interval was in the control group with 54.38 months and the lowest was for high-risk GTN with 30.80 months. No significant difference in smoking was observed between the case and control groups and between the GTD subgroups.

The demographic characteristics, the interval between the previous pregnancy and GTD, and β-hCG level rank were evaluated between the control group, mole hydatidiform with complete remission group, and patients who progressed to GTN (Fig. 1). The rank and mean of each group of the whole cohort were calculated. Age and abortion history were not significant between the groups whereas the gravidity and parity were higher, the interval was lower, and the β-hCG level was higher in the GTN progression group, which were expressed with a significance level of *p* as 0.015, 0.027, 0.027 and 0.001 respectively. The interval rank's mean was 35.25 for the GTN group, 45.46 for the mole group, and 52.66 for the control group. The β-hCG level rank's mean was 28.76 for the mole group, 35.67 for the control group, and 52.51 for the GTN group. When the GTN was further evaluated between the low-risk and high-risk groups, the only prognostic factor that remained was the lower interval between the previous pregnancy for the high-risk GTN which was 44.4 months for GTN cases with good prognoses and 23.9 months for GTN cases with poor prognoses ($p = 0.028$).

The immunohistochemical evaluation of p53, c-erbB-2, and Ki67 expression in the tissue and the *mdm2* gene expression in the blood sample was assessed between the case (whole GTD) and control groups (Figs. 2,3). Only p53 was found to be significantly higher in the GTD group. The p53 and c-erbB-2 positivity, and Ki67 and *mdm2* expressions were evaluated between the control group, hydatidiform mole in complete remission group, and GTN group. The results of Ki67 for each specimen were put into a rank scale from the lowest to highest expression and were numbered from 1 to 79 and the rank's mean was then calculated for each case subgroup and control group. The results of *mdm2* expression were also put into a rank from the lowest to the highest expression and the results were numbered from 1 to 71, the rank's mean was then calculated for each case subgroup and control group. The data demonstrated that p53 and c-erbB-2 positivity were significantly higher in the GTN group compared with the hydatidiform mole in the complete remission group (Table 3). Additionally, we also found that p-53 positivity was significantly higher in the high-risk GTN, while the *mdm2* expression level was significantly higher in the low-risk GTN subgroup (Table 4).

4. Discussion

Approximately 8–30% of GTD transforms into GTN [13]. Currently, serial serum β-hCG levels are used to predict GTN development, which is not accurate enough and often delays diagnosis [14]. Thus, there is still a lack of predictive and prognostic markers for GTN.

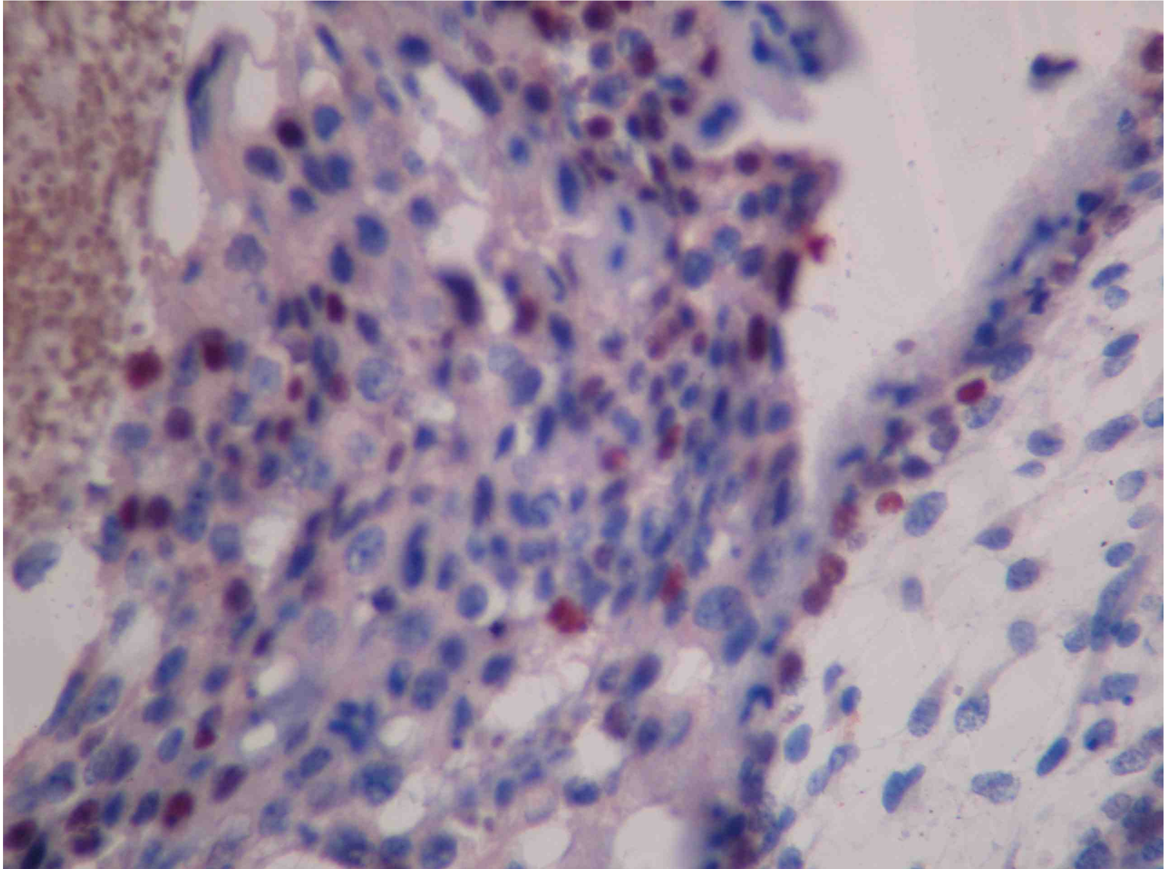

FIGURE 2. Significant nuclear immunoreactivity for p53 antibody in cytotrophoblasts.

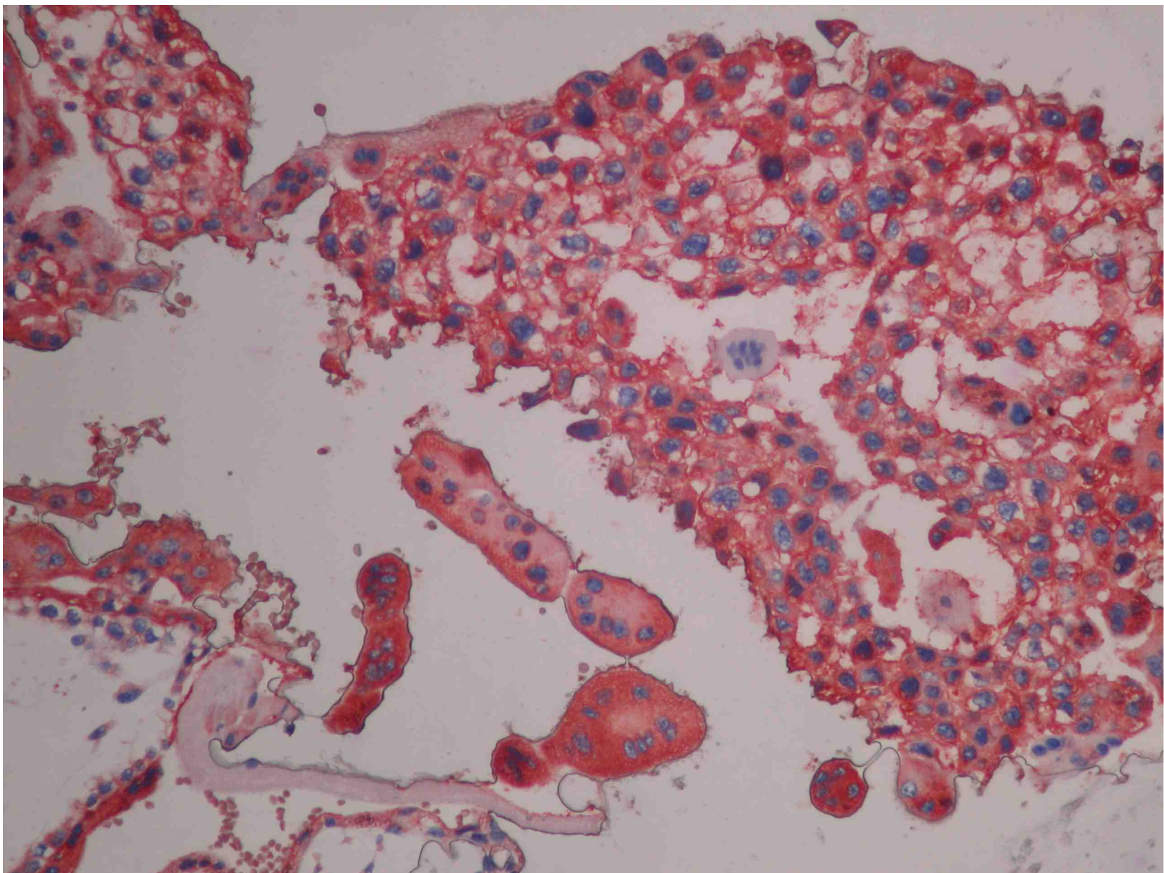

FIGURE 3. Significant membranous immunoreactivity for c-erbB-2 antibody in villous trophoblasts.

TABLE 3. Evaluation of p53, c-erbB-2, Ki67, and *mdm2* between the mole hydatidiform with complete remission, GTN, and control groups.

Markers	Control	Mole in Remission	Progression to GTN	*p* value
Mutant p53 complete positivity	4.80%	56.70%	82.10%	0.001
c-erbB-2 positivity	14.30%	10.00%	60.70%	0.001
Ki67 rank	34.74	42.18	41.61	>0.050
Blood sample mdm2 rank	30.5	35.62	40.04	>0.050

GTN: gestational trophoblastic neoplasia; mdm2: mouse double minute homolog 2; c-erbB-2: c-erythroblastic oncogene B-2.

TABLE 4. Evaluation of p53, c-erbB-2, Ki67, and *mdm2* between low-risk and high-risk GTN cases.

Markers	Low-risk GTN	High-risk GTN	*p* value
Mutant p53 complete positivity	65.30%	100.00%	0.014
p53	1.10 ± 1.14	1.80 ± 1.05	>0.050
c-erbB-2 positivity	71.40%	50.00%	>0.050
c-erbB-2	1.60 ± 1.28	1.10 ± 1.29	>0.050
Ki67 rank	21.60	31.90	>0.050
Ki67	21.60 ± 29.37	31.90 ± 25.97	>0.050
Blood sample mdm2	$6.71 \times 10^{-3} \pm 1.81 \times 10^{-2}$	$1.19 \times 10^{-3} \pm 2.75 \times 10^{-3}$	0.005

GTN: gestational trophoblastic neoplasia; mdm2: mouse double minute homolog 2; c-erbB-2: c-erythroblastic oncogene B-2.

Our results showed that the demographic characteristics were similar between the groups, indicating that this was a well-designed study. We found the interval after the last pregnancy was negatively correlated while the β-hCG level was positively correlated with GTN formation, concordant with the WHO/FIGO scoring system. This meant that the lower the interval after the last pregnancy, the higher the GTN formation risk; and the higher the β-hCG level, the higher the GTN formation risk for any GTD [4]. Age at diagnosis was not associated with GTN development. Interestingly, gravidity and parity were higher in the GTN group than in the other groups. We hypothesized that the reason might be due to every woman's increased risk of GTD with increased gestation. In addition, when GTN was divided into low and high-risk subgroups, this significance was lost. Further, the β-hCG difference between remission and progressive disease groups ceased when GTN was divided into the low and high-risk subgroups. Thus, the β-hCG level might be most important for predicting GTN but not the high or low-risk GTN.

p53 is a well-known tumor suppressor gene and its mutation is shown to be involved in several cancers' pathogenesis [21]. Several studies reported increased p53 expression in GTN compared with GTD with spontaneous remission [8, 15–20]. Our results showed that p53 expression was significantly increased in GTD rather than in the control group, indicating the relation with GTD formation. It was also significantly higher in the GTN progressing group rather than the spontaneous remission group, suggesting that *p53* may play a role in the malignant transformation of GTD, which was concordant with some other research as well [20–22]. Concordant with our study, Sun *et al.* [14] also revealed that the mutant type p53 expression was associated with the GTN development in hydatidiform moles with poor prognosis. Downregulation of a member of apoptosis-stimulating proteins of p53 (ASPPs), ASPP1 was demonstrated in GTD and lower ASPP1 was also shown in progression to GTN which may explain the pathogenesis through decreased apoptosis [23]. Another study revealed the overexpression of ASPP family inhibitory member iASPP, with a progressive increase from hydatidiform mole to choriocarcinoma [24]. Another study showed that a molecule DJ-1 as a regulator of Phosphatase and TENsin homolog deleted on chromosome 10 (PTEN), which is a frequently mutated tumor suppressor gene in carcinogenesis may play a role in apoptotic activity regulation of GTD in relation to PTEN and p53 [25]. Interestingly, p53 expression status but not p53 level was increased in the high-risk subgroup, showing that p53 might be used as a predictive indicator for GTN formation.

Research on *mdm2* has been gaining increasing attention, and this present study can be considered the first to evaluate its expression in blood samples. *Mdm2* gene expression was not different between the GTD and control group nor the GTD in remission and progression to the GTN group. However, as it was significantly different between the low- and high-risk subgroups, suggesting that blood *mdm2* expression might be a useful novel prognostic factor during the course of the disease. Previous studies only focused on immunohistochemical studies, with most unable to show a significant association with patients' prognoses [26, 27].

As a member of epidermal growth factor receptors (EGFRs) c-erbB-2 is involved in many carcinogenesis pathologies [21]. We did not observe a significant increase in c-erbB-2 in whole GTD cases but have found that the expression positivity was higher in the GTN group than the spontaneous remission of the mole group, which was similar to the previous studies which

showed that c-erbB-2 could be used in the early diagnosis of GTN progression; comparable with p53 [10, 20–22, 28]. A study also showed that the increased c-erbB-2 expression in combination with DNA hyperploidy was correlated with a more aggressive potential of GTD [29]. Another study also showed c-erbB-2 as a strong predictor of the progression of GTD to GTN [22]. Interestingly, another member of the EGRFs, c-erbB-3, was shown to be correlated with the development of persistent GTD [30].

Abnormal proliferation is a basic step in oncogenesis and Ki67 is a well-known proliferation marker [10]. For GTD, Ki67 has inconclusive results. Ki67 expression was found to be increased with GTD formation in some studies whereas others indicated that there was no such difference [10, 31–33]. The effect of Ki67 on GTN progression showed also inconclusive results where some studies showed increased expression in malignant formation whereas others were able to show no difference between GTD and GTN samples [31, 34]. Although the diagnostic or prognostic value of Ki67 was previously reported, Ki67 was not significantly different between any of the investigated groups in this study.

5. Conclusions

Immunohistochemical assessment of p53 and c-erbB-2 might serve as a predictor for GTD transformation into GTN, and blood mdm2 evaluation in GTN patients might be a prognostic factor between low- and high-risk GTN progression. Altogether, a low level of mdm2 within the leucocyte of a GTN patient may represent a poor prognosis and high- GTN progression risks, thus indicating the need for more aggressive treatment.

AUTHOR CONTRIBUTIONS

BO—methodology, software, validation, formal analysis, investigation, resources, data curation, writing—original draft, writing—review & editing, visualization, project administration, funding acquisition. AA—conceptualization, methodology, resources, writing—review & editing, supervision, funding acquisition. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Human Ethics Committee of the Cukurova University Medical Faculty (approval number: ÇU/2006/02-0175 01.02.2006), and the protocol conformed to the provisions of the Declaration of Helsinki. Informed consent was obtained from all patients who were alive, and from a first-degree relative for those who had deceased.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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