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PREDICTION OF MALIGNANT PROGRESSION OF MOLAR PREGNANCY BY P53 PROTEIN EXPRESSION IN MOLAR TISSUE

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ABSTRACT

Background: Gestational trophoblastic disease (GTD) is a heterogenous group of diseases characterized by abnormal proliferation of trophoblast tissue. The most common form of GTD is molar pregnancy. Some reports suggest that p53 expression in molar tissue that progressed to malignant disease was significantly higher than in hydatidiform mole (HM) that obtained spontaneous remission. **Objectives:** The aim of this study was to evaluate p53 protein expression in molar tissue for predicting malignant transformation of molar pregnancy. **Materials and Method:** This cross sectional analytical study was performed in the Department of Obstetrics and Gynecology, Chittagong Medical College Hospital. Consecutive sixty-three women with primary diagnosis of molar pregnancy was enrolled, who were subsequently had evacuation of HM. Histopathology was done and eight patients were excluded due to negative histopathology for HM and patients were followed up for 12 weeks to see the progression of the disease (spontaneous remission or GTN) by doing serum beta human chorionic gonadotrophin (βhCG). Five patients were excluded due to loss of follow up. Molar tissue blocks were collected and preserved and p53 immunohistochemistry was done. **Results:** Pre-evacuation levels of serum βhCG were >100000 mIU/mL and <100000 mIU/mL in 36 (72%) and 14 (28%) patients respectively. After 12 weeks 42 (84%) patients had <5 mIU/mL serum βhCG indicating spontaneous regression of the disease. 8 (16%) patients showed malignant transformation of the disease within this period. 31 patients (62%) had positive p53 expression and 19 (38%) had negative p53 expression. p53 had high sensitivity and negative predictive value (100%) but low specificity (45.2%) and positive predictive value (25.81%). The area under the Receiver Operating Characteristics curve for predicting malignant transformation was 0.938 with a proposed cut-off value of 42.5% stained cells which had sensitivity of 87.5% and specificity of 71.3%. **Conclusion:** Present study concludes that p53 protein expression was associated with malignant progression of molar pregnancy. Negative expression may predict spontaneous remission.

KEYWORDS: GTD, GTN, βhCG, p53.

MATERIALS AND METHODS

Study period: One year from 01.01.2020 to 31.12.2020

Study population: Women with molar pregnancy admitted in department of Obstetrics and Gynaecvology of CMCH during the study period.

Sampling technique: Consecutive sampling

Sample size:

The study aimed to determine the prediction ability of p53 protein expression in determining malignant transformation of molar tissue in patients with molar pregnancy. The sample size was calculated based on sensitivity and specificity as follow (Hoque 2019; Negida et al., 2019).

Sample size estimate for sensitivity:

$$n = \frac{Z^2 \times S_N \times (1 - S_N)}{d^2 \times Prevalence}$$

Where,

n = Sample size,

Z = Z-value of standard normal distribution at a given level of significance, 1.96 at 95% level.

S_N stands for sensitivity and was assumed to be 85.7% (Fayed et al., 2012).

Prevalence stands for prevalence of the disease (Malignant transformation in molar pregnancy) and was

assumed to be 40%=0.4 (Fayed et al., 2012)
d = acceptable error = 10% = 0.1
So,

$$Sample\ size\ for\ sensitivity = \frac{1.96^2 \times 0.857 \times (1-0.857)}{(0.1)^2 \times 0.4} = 117.69 \approx 118$$

Sample size estimate for specificity:

$$n = \frac{Z^2 \times S_p \times (1 - S_p)}{d^2 \times (1 - Prevalence)}$$

Where,
n = Sample size,
Z = Z-value of standard normal distribution at a given level of significance, 1.96 at 95% level.
S_p stands for specificity and is assumed to be 23.3%, (Fayed et al., 2012). Prevalence stands
for prevalence of the disease (Malignant transformation in molar pregnancy) and was assumed
to be 40%=0.4 (Fayed et al., 2012)
d=acceptable error=10%=0.1
So,

$$Sample\ size\ for\ specificity = \frac{1.96^2 \times 0.233 \times (1-0.233)}{0.1^2 \times (1-0.5)} = 137.31 \approx 137$$

Therefore, in this study, 118 molar pregnancies with malignant transformation and 137 molar pregnancies without malignant transformation should be included with a total sample size of 255 molar pregnancies. However, due to fund limitation and time limitation it was possible to enrolled 50 samples in the study.

Eligibility criteria

A. Inclusion criteria: Admitted patients were included if
1. They had molar pregnancy and underwent evacuation in CMCH.
2. The patients who wanted to participate in the research work and gave informed consent.

B. Exclusion Criteria: Patients with following characteristics were excluded
1. Patients with molar pregnancy with already evacuation done.
2. Patients with negative histopathology for molar pregnancy.
3. Patients who are haemodynamically unstable.
4. Patient's whose pre evacuation serum beta hCG was not available.
5. Women who already developed malignant disease.
6. Patients or guardian refused to participate in the study
7. Patients who discontinued follow up.

Data collection tool: Pre-designed case record form.

Procedure of data collection:
Eligible patients were informed about the nature of the study and an informed written consent was obtained prior to enrollment in the study. On admission detailed history, complete physical examination was performed. Preliminary laboratory investigation including pre evacuation serum βhCG was done. Total 63 samples were taken consecutively. Then evacuation of HM was done. Histopathology was done and blocks were preserved. Eight Patients were excluded due to negative histopathology for HM. Patients were followed up for 12 weeks to see the malignant progression of the disease or spontaneous remission by doing serum βhCG level as per guideline. Five patients were excluded due to loss of follow up. Patients were divided into two groups: (1) malignant disease or GTN group if serum βhCG level rises or do not change during study, (2) simple molar pregnancy (SMP) group whose serum βhCG were decrease gradually. p53 expression was determined from preserved block.

Histological Examination: The molar tissue was fixed in 10% formalin. Tissue processing was done in the Department of Pathology of Chittagong medical college following standard protocol. All the slides were stained with Hematoxylin and Eosin (H&E). The diagnosis was done based on criteria defined by various authors and most representative, paraffin embedded blocks were collected to perform immunohistochemical staining.

Immunohistochemical Examination: The most representative tumor tissue was chosen from each case and 3-4μm sections of formalin-fixed paraffin embedded tissue was taken. It was done in Dr. Lal Path Labs through Endeaver Health Care, Chattogram. The sections were mounted on poly –L-lysine coated slides dewaxed with xylene and gradually rehydrated. The activity of endogenous peroxidase was blocked by 30 min incubation in 1% H2O2. The sections were boiled for 10 min in a microwave oven, in Antigen Retrieval Solution (Dako Cytomation, Denmark) at 500W. This was followed by immunohistochemical reactions using monoclonal (ZJ11) mouse antibodies direct against p53 (Chemicon International, Temecula, CA, USA). The antibodies were diluted 1:100 in the Antibody Diluent, Background Reducing (Dako Cytomation, Denmark). The sections were incubated with an antibody for 1 hour at room temperature. Subsequently, they were incubated with biotinylated antibodies (15 min, room temperature) and with the streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB+, HRP, Dako Cytomation, Denmark). DAKO REAL™ EnVision™ (HRP RABBIT/ MOUSE) (ENV) was used as a chromogen, employing 7 minute incubation at room temperature. All the sections were counter stained using Meyer's hematoxylin. Routine positive control will also be processed as same manner.

As scoring algorithms of the p53 immunohistochemistry have not been optimized and standardized, nuclear staining was considered immune positive. Adopted the German semiquantitative scoring system in considering the staining intensity and extension of stained cell. The final immunoreactive score was determine by multiplying the intensity and extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 12.

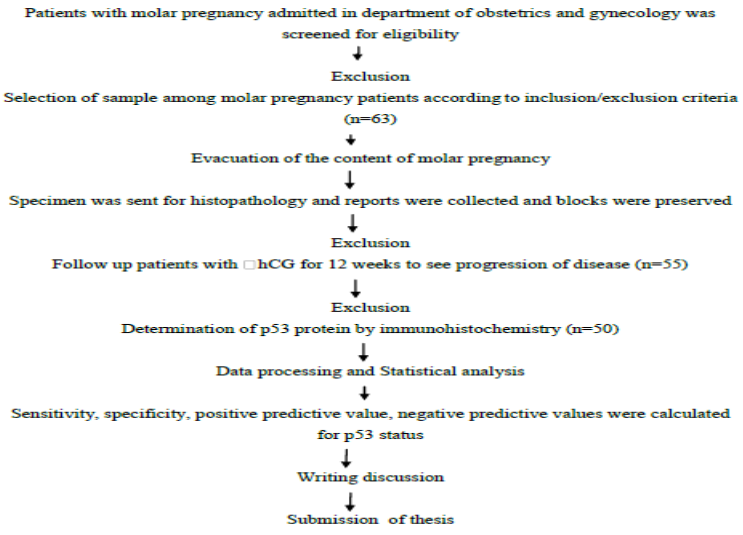
Intensity Score	Staining pattern
0	No staining (negative)
1	Weak staining
2	Moderate staining
3	Strong staining
Score	**Extent of stained cells(percentage)**
0	Non immunoreactive
1	Immunoreactive in<5% cells
2	Immunoreactive in 5-9 % cells
3	Immunoreactive in 10-49% cells
4	Immunoreactive in ≥ 50% cells
Final Score	**P53 Expression**
0-3	Negative
4-12	Positive

Data analysis: After collection data were entered into Microsoft Excel worksheet to generate a master sheet. Next, they were fed into computer based software SPSS-version 23 for processing and analysis. Variables were expressed as frequency and percentages. Chi-square test or Fisher's exact test was used to compare categorical variables between groups. A p-value less than 0.05 was considered statistically significant. To estimate a cut off for percentage of positive immunostained cells ROC (receiver operating characteristic) curve analysis was applied to evaluate the risk of transformation of molar pregnancy to gestational trophoblastic neoplasia. Keeping serum □hCG level as gold standard, sensitivity, specificity, positive predictive value and negative predictive value of p53 were calculated and 95% confidence levels were estimated.

Clarification of ethical issue:

i. Voluntary informed consent was taken from each patient and/or legal guardian of patients enrolled in the study.
ii. All measures were taken to preserve participant anonymity and privacy. Personal information of any of them was not handed over to any third party without their consent.
iii. The enrolled patients were treated as per treatment guideline for the disease of research interest by the concerned medical personnel. Researcher did not participate in treatment process of participants.
iv. Financial support was not provided to any research participant for being a subject in the research work. All diagnostic and therapeutic interventions including evacuation of pregnancy products, serial weekly measurement of serum βhCG, and histological evaluation for their primary diagnosis (complete or partial molar pregnancy) were performed according to indications for patients with molar pregnancy diagnosis. Immunohistochemistry expenses were covered by the research budget.
v. All patients and/or legal guardian were informed about the nature and purpose of the study. They got informed that their participation in the coming study research would not only benefit them but also whole community as well.
vi. Evaluation of each research participant was done thoroughly.
vii. Ethical clearance was taken from Ethical Review Committee of Chittagong Medical College.

Study flow chart



RESULT

Table I: Age distribution of the patients (n=50).

Age group	Frequency (Percentage)
16-25 years	33 (66.0)
25-40 years	15 (30.0)
>40 years	2 (4.0)

Table I depicts that, majority of the patients were <25 years (66%), followed by 30% in the age group of 25-40 years and only 2(4%) patients were above 40 years.

Table II: Obstetric and Gynecological characteristics of the patients (n=50).

Variables		Frequency (percentage)
Age at menarche	<14 years	38 (76.0)
	>14 years	12(24.0)
Married for	<5 years	24 (48.0)
	5-10 years	13 (26.0)
	>10 years	13 (26.0)
Gravida	Primi	16(32.0)
	1-2	27(54.0)
	>4	7 (14.0)
Parity	Nulliparous	23(46.0)
	1-2	20(40.0)
	≥3	7 (14.0)
H/O abortion		9 (18.0)

Obstetrical and gynaecological characters of the patients are shown in table II. It depicts that, age of menerchae was <14 years in majority of the patients (76%). Only 9 (18%) patiets had history of abortion. 46% of the patients were nulliparous.

Table III: Immunohistochemical analysis of p53 in the molar tissue (n=50).

Parameters	Frequency (Percentage)
Staining pattern	
Weak staining	8 (16.0)
Moderate staining	23 (46.0)
Strong staining	19 (38.0)
Extent of stained cell	
0% stained cells	2 (4.0)
<5% stained cells	18 (36.0)
5-9% stained cells	11 (22.0)
10-49% stained cells	18(36.0)
≥50% stained cells	1(2.0)

In majority of the cases staining pattern was either moderate (46%) or strong (38%). Regarding percentage of stained cells 36% of the cases had <5% and 10-49% cells stained respectively. Only 2 (4%) cases and 1 (2%) case respectively took no staining and ≥50% cell staining (Table III)

Table IV: Association between pre-evacuation βhCG level and p53.

Immunostaining (n=50)			
p53 immunostaining	pre-evacuation βhCG level		P value
	<100000 mIU/mL (n=14)	>100000 mIU/mL(n=36)	
Positive	6 (42.9)	25 (69.4)	0.082*
Negative	8 (57.1)	11 (30.6)	

***P value was obtained from Fisher's exact test**

Out of 14 patients with pre-evacuation βhCG level <100000 mIU/mL 6 (42.9%) had p53 positive immunostaining and in 36 patients with pre-evacuation βhCG level >100000 mIU/mL 25 (69.4%) had p53 positive immunostaining. The association was not statistically significant (p=0.082) (Table IV).

Table V: Association between diseases progression and p53 immunostaining p53 immunostaining

	Progress (n=8)	Regressed (n=32)		
Positive	8 (100.0)	23 (54.8)	31 (62.0)	
Negative	0 (0)	19 (45.2)	19 (38.0)	0.018*
Total *p value was obtained from Fisher's exact test	8 (16.0)	42 (84.0)	50 (100.0)	

Malignant transformation of the HM

Statistic	Value	95% CI
Sensitivity	100.00%	63.06% to 100.00%
Specificity	45.24%	29.85% to 61.33%
Positive Likelihood Ratio	1.83	1.39 to 2.40
Negative Likelihood Ratio	0.00	---
Disease frequency	16.00%	7.17% to 29.11%
Positive Predictive Value	25.81%	20.90% to 31.41%
Negative Predictive Value	100.00%	---
Accuracy	54.00%	39.32% to 68.19%

Total (n=50)
P val
*P value was obtained from Fisher's exact test

Table V shows that, p53 had high sensitivity and negative predictive value (100%). But the specificity and positive predictive value was low, 45.2% and 25.81% respectively.

By plotting a ROC curve a cut-off value for the percentage of cells stained by p53 was establish as 42.5% to assess hydatidiform mole progression to gestational trophoblastic neoplasia (Fig. 4). Adopting this cutoff in this test presents: sensitivity of 87.5%, specificity of 71.3%.

Table VI: Validity of measured p53 staining in predicting the malignant transformation by the cut-off value of 42.5% stained cells.

Statistic	Value	95% CI
Sensitivity	87.50%	47.35% to 99.68%
Specificity	71.43%	55.42% to 84.28%
Positive Likelihood Ratio	3.06	1.78 to 5.28
Negative Likelihood Ratio	0.17	0.03 to 1.11
Disease frequency	16.00%	7.17% to 29.11%
Positive Predictive Value	36.84%	25.27% to 50.16%
Negative Predictive Value	96.77%	82.60% to 99.48%
Accuracy	74.00%	59.66% to 85.37%

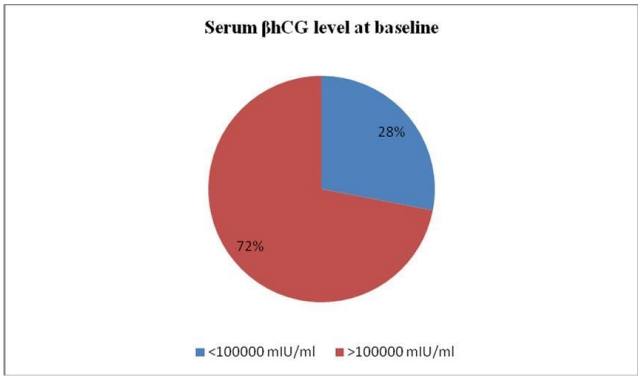


Figure 1: Distribution of the patients according to their baseline serum βhCG level (n=50).

Pre-evacuation βhCG level was >100000 mIU/mL in 36 (72%) patients and it was <100000 mIU/mL was in 14 (28%) patients (Figure 1).

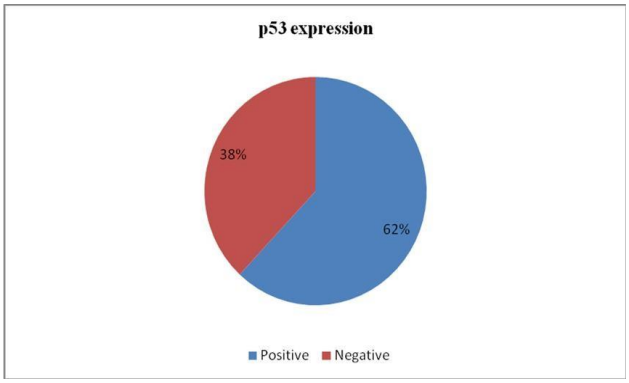


Figure 2: p53 expression status in the patients (n=50).

Out of 50 patients 31 (62%) had positive p53 stating and 19 (38%) had negative p53 staining (Figure 2)

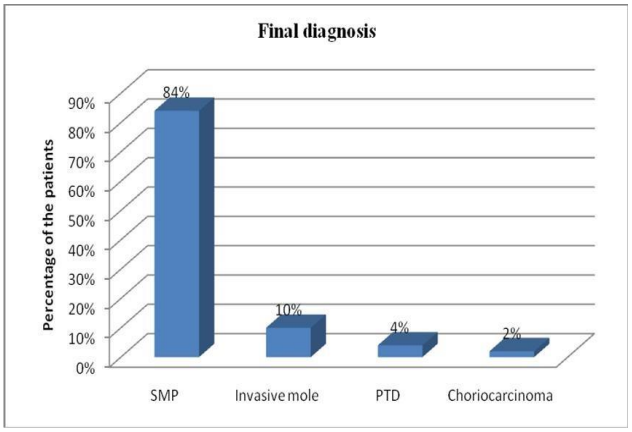
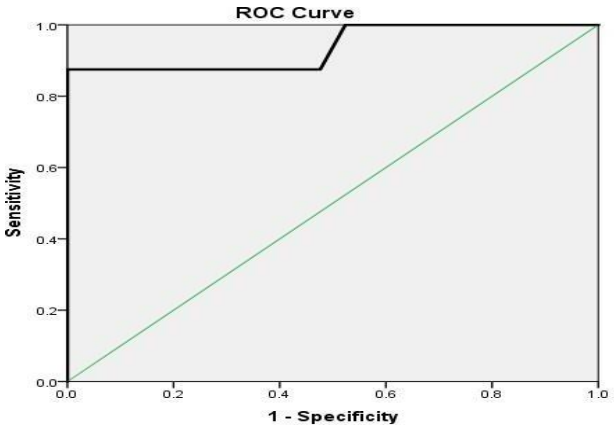


Figure 3: Final diagnosis of the patients (n=50).

Figure 3 shows out of 50 patients with primary diagnosis of molar pregnancy, 1(2%), 2(4%) and 5(10%) patients developed choriocarcinoma, persistent trophoblastic disease and invasive mole respectively. Remaining 42(84%) patients had spontaneous remission. The patient with choriocarcinoma presented with high rise of βhCG within a short period after evacuation. Hysterectomy was done and on histopathology choriocarcinoma was diagnosed. Seven patients were presented with rising or plateaued βhCG. Ultrasonography revealed uterine invasion in five cases and those were diagnosed as invasive mole. Those without uterine invasion was diagnosed as PTD.



Area under the curve (AUC)	95% Confidence Interval		P value
	Lower limit	Upper limit	
0.938	.820	1.000	<0.001

Figure 4: Estimation of ideal cut-off value of percentage of cells with stained by p53 in the evaluation of HM transformation.

DISCUSSION

The current clinical method to predict the malignant transformation of HM is through regular follow-up of serum βhCG after evacuation, but this method requires patients to have good compliance and clinically reliable βhCG testing conditions. Due to this follow-up schedule some of the patient only found after the malignant transformation or after the lungs and other distant metastases had appeared. Therefore, looking for early detecting molecular markers for predicting progression of HM to GTN provides a theoretical basis for prophylactic chemotherapy, in addition, it may provide a new therapeutic target for GTN to reduce tumor resistance and relapse. The present study attempted to test the expression pattern and the possible predictive value of expression of p53 in the malignant transformation of molar pregnancy. In the present study p53 expression was immunohistochemically assessed in 50 patients with simple molar pregnancy and the patients were prospectively followed for 12 weeks to observe progression of the disease. It was observed that *p53* genes had higher expressions in patients with malignant transformation in comparison with simple molar patients with significant difference.

In the present study p53 expression was positive in 62% (31/50) of the cases. Previous findings supported that p53 is a useful marker to differentiates hydropic abortion from molar pregnancy. Increased over expression of p53 in GTDs seems to correlate with the higher trophoblastic proliferation rate found mainly in complete and invasive HMs (Kale et al. 2001 ; Kheradmand et al.,2018; Abdulhameed et al., 2018; Khooej et al., 2019) βhCG is an excellent biomarker of disease progression, response, and subsequent post treatment surveillance. A plateaued or rising βhCG level enables the early detection of progression of CHM and PHM to GTN that occurs in 15%–20%, and 0.5%–5% of cases, respectively (Lurain et al., 2010; Sebire & Seckl 2010). In the present study, out of 50 patients frequency of a persistent mole that needed chemotherapy was 8 (16%). Fayed et al. (2012) observed a higher in (about 40%), in a tertiary referral center of Egypt.

In terms of the prediction of disease progression, results of the current study showed that tumor suppressor protein p53 had good validity, showing a relatively high sensitivity (100%) and NPV (100%) and poor specificity (45.24%) and PPV (25.81%). This was comparable with the findings of Fayed et al., (2012) where p53 had a relatively high sensitivity (85.7%) and NPV (71.4%) and poor specificity (23.3%) and PPV (42.1%) to diagnose mole that had malignant transformation. This results indicated that, p53 markers test is good if negative, but poor positive tests. The higher sensitivity indicates that, we can completely exclude the need for chemotherapy in the absence of expression of p53 marker.

An attempt was taken to determine the cutoff value of p53 marker having the highest sensitivity and specificity which might increase the risk of progression of a molar pregnancy by ROC curve analysis. It was observed that, a value of 42.5% p53 stained cell was the most appropriate cutoff value to assess HM progression to GTN with a sensitivity of 87.5% and specificity of 100%. Hasanzadeh et al., (2016) found the positive predictive values of 90% and 88.8% when 5.5% and 2.5% of cytotrophoblast and syncytiotrophoblast with positive nuclear immunoactivity were used as the cut off respectively. Cut-off value was 40.1% stained cells to assess hydatidiform mole progression to GTN with sensitivity of 66.7%, specificity of 65.6%, positive predictive value of 68.6% and negative predictive value of 63.6% in the study of Yazaki-Sun et al., (2006). The area under the curve was 0.938 in the present study and the corresponding figure was 0.917 according to Hasanzadeh et al., (2016) and 0.670 in the study of Yazaki-Sun et al., (2006) indicating comparable and high predictive utility. Few studies assessed the predictive ability of combination of p53 with other markers. Like Sun et al., (2016) reported that, the combination of maspin negative expression with p53 positive expression had an 84% specificity value, 76% positive predictive value and 70% negative predictive value for the development of GTN. Fayed et al., (2012) reported that, concomitant negative expression of both C-erbB-2 and p53 markers was associated with spontaneous remission in 100% of our studied group (NPV 100%).

At present, serial serum β-HCG levels are the standard in predicting the development of GTN. However, in addition to being time consuming and inconvenient, the diagnosis is typically delayed when using this method (Ngu & Chan 2014). In this aspect the present study findings have great clinical utility. This finding, if confirmed by further larger studies, may allow a shorter follow-up period and it may be possible to guide the selection of high-risk patients and initiation of prophylactic chemotherapy. Based on these results further collaboration of pathologists and gynecologists would be suggested to establish comprehensive guidelines for early diagnosis of malignant progression of molar pregnancies.

CONCLUSIONS

In conclusion, results and observation of the present study indicated that over expression of p53 was associated with the malignant transformation of molar pregnancy. It was encountered that, high expression of p53 in trophoblastic cells could predict GTN in early stages. Negative expression may predict spontaneous remission. However, studies till date are not consistent in this regards and further studies are required to recommend the routine use of this marker for prognostification following molar evacuation.

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