Expression patterns of maspin and mutant p53 are associated with the development of gestational trophoblastic neoplasia

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Abstract. Gestational trophoblastic disease (GTD) is a group of conditions that originate from the abnormal proliferation of trophoblastic cells. GTDs encompass hydatidiform moles (HMs) and gestational trophoblastic neoplasia (GTN). GTNs are a group of malignant diseases that require chemotherapy, or more aggressive treatment. There is a requirement for more tumor markers to predict the development of GTN from HMs. The current study evaluated the expression of maspin and tumor protein p53 (p53) in GTD, and their role in predicting the development of GTN. Expression of maspin and mutant p53 (m-p53) was detected by immunohistochemistry in 48 normal first trimester placentas, matched for gestational age to 49 HMs that regressed, 39 malignant HMs and 11 invasive moles or choriocarcinomas. Spearman’s rank correlation analysis and logistic regression were performed on the expression patterns of maspin and m-p53, and on the clinical prognostic factors in GTD. Compared with normal placenta levels, the expression levels of maspin were decreased, whereas the expression levels of m-p53 were increased in GTDs (P<0.05). The expression levels of maspin and m-p53 in complete and partial HMs were not significantly different (P>0.05). In HMs, maspin expression was inversely correlated with serum β human chorionic gonadotropin, uterine size and diameter of theca-lutein cysts; however, m-p53 expression demonstrated a positive correlation with these factors (all P<0.05). Compared with the high-risk metastatic group (FIGO score ≥7), the low-risk group (FIGO score <7) exhibited a higher rate of positive maspin expression (P=0.041), and the frequency of positive m-p53 expression was significantly higher in patients with an advanced FIGO stages (FIGO stage ≥III) compared with patients in early stages (FIGO stage ≤II; 87.9 vs. 58.8%; P=0.019). The combination of maspin negative expression with m-p53 positive expression had an 84% specificity value, 76% positive predictive value and 70% negative predictive value for the development of GTN. In conclusion, maspin-negative and m-p53-positive expression is associated with the development of GTN in HMs.

Introduction

Gestational trophoblastic disease (GTD) is a group of conditions that originate from the abnormal proliferation of trophoblastic cells, which derive from the trophectoderm, the outer layer of the blastocyst that would normally develop into the placenta during pregnancy (1,2). GTDs encompass hydatidiform moles (HMs; complete and partial), invasive moles (IMs), choriocarcinoma (CCA), placental-site trophoblastic tumors and epithelioid trophoblastic tumors. HMs are a type of aberrant human pregnancy with abnormal embryonic development. HMs occur in ~1:600 pregnancies in the United Kingdom (1), with even higher rates in the Middle East, Latin America, Africa and the Far East (2-5). The majority of HMs will spontaneously regress following suction evacuation; however, 8-30% of HMs persist after evacuation and develop into gestational trophoblastic neoplasia (GTN), requiring chemotherapy (6).

Although GTN typically responds well to chemotherapy, chemoresistant cases still exist, even despite advances in chemotherapy. Since the introduction of chemotherapy, reliable measurement of serum β human chorionic gonadotropin (β-hCG) levels and individualized risk-based therapy into the management of GTN, the majority of low-risk and 80%...
of high-risk GTN cases are curable (6). However, 15-25% of high-risk GTNs develop resistance to chemotherapy, or relapse following the completion of initial therapy, necessitating salvage combination chemotherapy (7). At the opposite end of the spectrum, a proportion of patients with GTD have persistently low levels of β-hCG without clinical or radiological evidence of disease, a condition called quiescent GTD. While there is a growing understanding of the molecular biology of GTD, the precise molecular signaling pathways underlying the development of GTD require further exploration (6,8,9). More tumor markers to predict the development of GTN from GTD are required. Thus, the current study evaluated maspin and tumor protein p53 (p53) expression in GTD.

Maspin was identified in 1994 by subtractive hybridization analysis of normal mammary tissue and breast cancer cell lines (10). A previous study demonstrated that maspin is a multifaceted protein, interacting with a diverse group of intercellular and extracellular proteins responsible for regulating cell adhesion, motility, apoptosis and angiogenesis, and is critically involved in mammary gland development (11). The expression of maspin has been observed to inhibit tumor cell invasion and metastasis in breast, prostate, ovarian, colorectal and several other types of cancer (12-15). p53 has been identified as a regulator of maspin expression in breast, lung and colorectal cancer cell lines (13-15). In mammary epithelial cells, the wild-type p53 (wt-p53) binds to the promoter of maspin in breast, lung and colorectal cancer cell lines (13-15). In mammary epithelial cells, the wild-type p53 (wt-p53) binds to the promoter of maspin and activates its expression, leading to inhibition of cellular invasion and migration (16). These findings suggest that the association between maspin and p53 may have some clinical significance in the development of malignant disease.

Using placental tissue from gestational age-matched, normal first or early-second trimester pregnancies as a control, the present study examined the expression of maspin and mutant p53 (m-p53) in GTD and evaluated its potential prognostic value.

Materials and methods

Clinical samples. A total of 99 formalin-fixed, paraffin-embedded (FFPE) placental tissue blocks were used in the present study, including 49 HMs that regressed (rHMs), 39 malignant HMs (mHMs) that subsequently progressed to GTN after 2 years (determined in follow-up), 4 IMs and 7 CCAs. Tissue specimens were collected from the Fujian Maternity and Children Health Hospital (Fuzhou, China). Study approval was obtained from the Institutional Research Board of FWCH. As a control, fresh placental tissue samples were collected from 48 normal first or early-second trimester pregnancies, matched by gestational ages to the molar specimens. GTN was diagnosed based on a plateau in β-hCG levels for 4 measurements over 3 weeks, or by a rise in β-hCG levels for 3 consecutive measurements over 2 weeks when pregnancy was excluded. Further information on the study population is listed in Table I. The International Federation of Gynecology and Obstetrics (FIGO) stage (2000) and FIGO risk prognostic factor scores (2002) were collected for all patients (17).

Serum β-hCG assay. Architect™ total β-hCG reagent kit (Abbott Laboratories, Lake Bluff, IL, USA) was used to perform serum β-hCG quantitative measurement in an ARCHITECT I 2000SR immunoassay analyser system (Abbott Laboratories) for 45 min.

Immunohistochemical staining. Immunohistochemistry using placental tissue was performed as previously described (18). Briefly, serial 5-µm FFPE tissue sections were cut and de-waxed in xylene and rehydrated through graded ethanol, followed by Tris-buffered saline (TBS). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 5 min. For antigen retrieval, the tissue sections were boiled at 96-98°C in 0.01 M sodium citrate buffer in a microwave oven for 5 min and cooled to room temperature. Non-specific binding was blocked by incubating the tissue sections with Protein Block Serum-Free (Dako North America, Inc., Carpinteria, CA, USA) for 5 min. Immunohistochemistry was performed using a rabbit anti-human maspin antibody (1:200 dilution; cat. bs-0792R; Bioss, Beijing, China) and a mouse anti-human m-p53 antibody (1:250 dilution; cat. sc-126; Santa Cruz Biotechnology, Dallas, USA). A PV-9000 2-Step Plus® Poly-horseradish peroxidase (HRP) Anti-Mouse/Rabbit IgG Detection System (Golden Bridge International, Mukilteo, WA, USA) was used to detect the target proteins. Briefly, the tissue sections were incubated with the rabbit anti-human maspin antibody or the mouse anti-human m-p53 antibody at room temperature for 1 h, followed by washing with phosphate-buffered saline for 2 min a total of 3 times. Subsequently, the sections were incubated with polymer helper (provide by the PV-9000 2-Step Plus® system) at room temperature for 20 min, and HRP labeled poly peroxidase-anti-mouse/rabbit IgG (provided by the PV-9000 2-Step Plus® system) at room temperature for 30 min. The color was developed and visualized by using an EnVision DAB Detection System (Dako, Glostrup, Denmark). Negative controls were prepared by replacing the primary antibody with TBS. Positive controls were prepared using a known maspin-positive first-trimester trophoblastic tissue sample, and breast cancer tissue with a known m-p53 gene, which were set up by the Laboratory of Pathology Department, Fujian Maternity and Children Health Hospital (Fuzhou, China).

Assessment of immunohistochemical staining. Two independent pathologists assessed the immunostaining. A total of 4 fields/section were selected and 10 images/field were captured at random using a light microscope (BX-51; Olympus Corporation, Tokyo, Japan) with a digital camera (DP70; Olympus Corporation). Staining intensity was scored on an arbitrary scale: 0, no immunoreactivity; 1, weak; 2, moderate; and 3, intense. In each image, 100 cells were counted and recorded. The percentage of positive cells was graded as follows: 0, negative; 1, <33%; 2, 33-67%; and 3, >67%. The overall immunoreactivity was determined through the multiplication of the above two parameters to give a composite ‘histoscore’ with a maximum score of 9 (18,19). A histoscore >3 was defined as representative of positive expression for maspin or P-53.

Statistical analysis. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). All parametric results, for age, β-hCG level, gestation time, production
time and immunohistochemical scores are expressed as the mean ± standard deviation and were compared by analysis of variance. The rate of positive expression was expressed as % and compared by χ² and Fisher's exact test. Spearman's rank correlation analysis, logistic regression and multivariable linear regression analysis were also used. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of maspin and m-p53. The expression of maspin in normal first-trimester placental tissue, HMs and IMs was localized to the cytoplasm of the trophoblastic cells, with markedly higher expression in cytotrophoblasts compared with syncytiotrophoblasts (Fig. 1). From normal first-trimester placentas to rHMs, mHMs and IM/CCAs, positive maspin expression decreased significantly (χ²=30.34; P<0.001; Fig. 2A). Compared with the normal first-trimester placenta, positive expression of maspin was significantly less frequent among rHMs (χ²=5.81; P=0.016), mHMs (χ²=18.86; P<0.001) and IM/CCAs (χ²=22.82; P<0.001). Compared with rHMs, positive maspin expression was significantly lower in mHMs (χ²=7.52; P=0.006) and IM/CCAs (χ²=11.47; P<0.001). No significant differences were identified in maspin expression between mHMs and IM/CCAs (χ²=2.93, P=0.087). Immunostaining performed on 7 cases of CCA revealed that the tumor cells did not stain for maspin. Conversely, the expression of m-p53 was observed in the nucleus of cytotrophoblastic cells and in intermediate trophoblast populations within the placental tissue. A step-wise increase in positive m-p53 expression was observed from the normal first-trimester placenta to the rHMs, mHMs and IM/CCAs (χ²=24.18; P<0.001; Fig. 2B). Compared with the normal placenta, the positive expression of m-p53 was significantly higher in the rHMs (χ²=4.64; P=0.031), mHMs (χ²=17.13; P<0.001) and IM/CCAs (χ²=15.12; P<0.001). Compared with rHMs, significantly higher rates of positive m-p53 expression were observed in mHMs (χ²=6.75; P=0.009) and IM/CCAs (χ²=7.01; P=0.016). There was no significant difference in m-p53 expression between the mHMs and IM/CCAs (χ²=2.02; P=0.242).

Expression of maspin and m-p53 in complete moles and partial moles. Of the 49 rHMs, 35 were complete HMs and 14 were partial HMs. Of the 39 mHMs, 23 were complete HMs and 16 were partial HMs. The frequencies of positive expression of maspin and m-p53 among the complete HMs [55.17% (32/58 cases) and 60.34% (35/58 cases), respectively] and partial HMs [46.67% (14/30 cases) and 56.67% (17/30 cases), respectively] were similar. Additionally, no significant differences were identified in the expression of maspin and m-p53 between complete and partial moles in rHMs or mHMs (all P>0.05; Fig. 3).

Association between the expression of maspin and m-p53 and clinical risk factors. Among the 88 cases of HMs, 39 developed into GTN within the 2-year follow-up period. The present study analyzed the association between the risk factors of HM and the expression of maspin and m-p53 (Table II). The expression of maspin was not associated with age. By contrast, it was significantly decreased in the serum β-hCG >1x10⁶ mIU/ml group vs. the ≤1x10⁶ group (χ²=15.88; P<0.001), the large-for-date uterine size group vs. the smaller group (χ²=13.00; P<0.001), and the ovarian theca-lutein cysts >6 cm group vs. the ≤6 cm group (χ²=7.57; P=0.006). Similarly, the expression of m-p53 did not differ between age groups. However, there was an observed increase in the expression of m-p53 in patients with serum β-hCG levels >1x10⁶ mIU/ml (χ²=7.65; P=0.006), large-for-date uterine size (χ²=5.14; P=0.023) and ovarian theca-lutein cysts >6 cm (χ²=6.29; P=0.012).

Expression of maspin and m-p53 in HMs. Of 88 HMs, 24 cases were positive for the expression of maspin and negative for the expression of m-p53, 30 cases were negative for the expression of maspin and positive for the expression of m-p53, 22 cases were positive for the expression of both maspin and m-p53, and 12 cases were negative for the expression of both maspin and m-p53. Spearman's rank correlation analysis revealed a significant inverse correlation between the expression of maspin and m-p53 (r=-0.240; P=0.005). Maspin was inversely correlated with serum β-hCG levels (r=-0.425; P<0.001), uterine size (r=-0.384; P=0.001) and diameter of theca-lutein cysts (r=-0.271; P=0.011). By contrast, the expression of m-p53 was revealed to have a significant positive correlation with serum β-hCG levels (r=0.425; P=0.005), uterine size (r=0.242; P=0.023) and diameter of theca-lutein cysts (r=0.172; P=0.024; Table III).

Table I. Clinicopathological characteristics of the patients (n=147).

| Variable     | Normal (n=48) | C (n=35) | P (n=14) | C (n=23) | P (n=16) | GTN (n=11) | P-value* |
|--------------|---------------|----------|----------|----------|----------|------------|----------|
| Age, years   | 26.05±4.61    | 27.06±9.39 | 26.43±8.18 | 29.13±7.81 | 33.06±11.60 | 31.55±4.87 | 0.055    |
| β-hCG level  | -             | 2.63±3.16 | 3.20±4.02 | 4.68±4.80 | 4.61±5.73 | 4.12±5.02 | 0.064    |
| Gestation    | 1.92±0.88     | 2.46±1.07 | 2.29±0.73 | 2.57±0.90 | 2.38±0.89 | 2.45±0.52 | 0.294    |
| Production   | 0.50±0.66     | 0.46±0.66 | 0.43±0.51 | 0.57±0.51 | 0.625±0.719 | 0.72±0.47 | 0.055    |

*Analysis of variance was used to compare the parametric data. HMs, hydatidiform moles; C, complete moles; P, partial moles; GTN, gestational trophoblastic neoplasia; β-hCG, β human chorionic gonadotropin; -, data not available.
Expression of maspin and m-p53 in GTNs. Maspin and m-p53 expression was analyzed in 50 cases of GTN, including 39 HMs and 11 IM/CCAs. Maspin expression was significantly higher in the low-risk group compared with the high-risk group (37.8% in patients with FIGO scores ≤7, vs. 7.7% in patients with scores ≥7; P=0.041). m-p53 expression was significantly higher in advanced stages compared with early stages (87.9% in FIGO stage III and IV vs. 58.8% in stage I and II; P=0.019; Table IV).

Prognostic value of the expression of maspin and m-p53 in the development of GTNs. Logistic regression analysis demonstrated that the expression of maspin in HMs was associated with a lower risk of developing GTNs (Table V; odds ratio (OR)=0.305; P=0.011), whereas m-p53 expression was associated with a greater risk of developing GTNs (OR=3.189; P=0.017). Of the classic prognostic factors for the development of HMs into GTNs, such as age (>40 or <20 years), high serum β-hCG levels, larger uterine size and bigger theca-lutein cysts,
only age and serum β-hCG levels demonstrated significance, and therefore were used in the regression model.

As demonstrated by the regression model, maspin and m-p53 expression predict the risks of developing GTN (Table VI). Absence of maspin expression had a 74.10% sensitivity and 65.31% specificity, whereas the presence of m-p53 expression had a 74.36% sensitivity and 53.06% specificity in predicting the development of GTN. Patients that were both negative for maspin and positive for m-p53 had the highest risk of developing GTN, with a specificity of 83.67%, a positive predictive value of 75.68% and a negative predictive value of 70.21%.

Figure 1. Immunohistochemical staining of maspin and m-p53. Normal placenta tissue showing (A) marked expression of maspin and (B) near absent expression of m-p53. Regressive HM showing (C) low expression of maspin and (D) low expression of m-p53. Malignant HM showing (E) low expression of maspin and (F) low expression of m-p53; invasive mole/choriocarcinoma tissue showing (G) absent expression of maspin and (H) marked expression of m-p53. Nuclei and cytoplasmic staining is observed in the villous cytotrophoblasts and syncytiotrophoblasts, however, the majority of maspin expression is observed in the cytoplasm of cytotrophoblasts. Following staining with the DAB system, a yellow to dark brown color for maspin expression was observed in the cytoplasm of cells, while it was observed in the nucleuses of cells for m-p53 staining. Magnification, x100. HM, hydatidiform mole.

Figure 2. Expression of (A) maspin and (B) m-p53 in various types of tissue. (A) Compared with the normal first-trimester placenta, the rate of positive expression of maspin was significantly lower in rHMs, mHMs and IM/CCA. Compared with rHMs, the positive expression of maspin was significantly lower in mHMs and IM/CCAs. There was no significant difference between the frequency of positive maspin expression in mHMs and IM/CCAs. (B) Compared with the normal placenta, the positive expression of m-p53 was significantly higher in rHMs, mHMs and IM/CCAs. Compared with rHMs, the positive expression of m-p53 was significantly higher in mHMs and IM/CCAs. The expression of m-p53 in mHMs and IM/CCAs was not significantly different. m-p53, mutant tumor protein p53; rHMs, regressive hydatidiform mole; mHMs, malignant hydatidiform mole; IM/CCA, invasive mole/choriocarcinoma.
Discussion

Worldwide, HMs occur in 0.5-3.8 per 1,000 pregnancies, with Asian populations experiencing a higher incidence of GTD compared with Western populations (2-5). Approximately 8-30% of HMs may develop into a malignant disease (5-6). 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 (6,11). Although the majority of GTNs are curable, ~25% of GTNs develop resistance to chemotherapy or relapse following completion of initial therapy (6,7,11). At present, there is a relative lack of predictive markers for GTN. Novel markers include telomerase activity, apoptotic activity and expression of Siglec-6, all of which have been associated with the development of GTN from HMs (20-22); however, these correlations have yet to be conclusively demonstrated.

Maspin is a tumor suppressor that has been demonstrated to inhibit trophoblastic invasion (11-13,23). The present study...
was conducted to investigate the hypothesis that HMs with downregulated maspin expression may have a higher invasive potential and a higher propensity for developing GTN. The p53 gene was another good candidate due to its correlation with maspin expression (14) and its expression in cytotrophoblasts (24). Whether these results regarding maspin and p53 have potential clinical applications in terms of prognostic value requires further investigation.

As demonstrated by immunohistochemistry, maspin was expressed in the cytoplasm and nucleus, but mostly in the cytoplasm of trophoblastic cells, with greater expression in cytotrophoblasts than in syncytiotrophoblasts (24). Whether these results regarding maspin and p53 have potential clinical applications in terms of prognostic value requires further investigation.

As demonstrated by immunohistochemistry, maspin was expressed in the cytoplasm and nucleus, but mostly in the cytoplasm of trophoblastic cells, with greater expression in cytotrophoblasts than in syncytiotrophoblasts. However, Li et al (23) reported expression of maspin in the nuclei of GTDs. The results of the present study were concordant with those of several previous studies conducted on mammary, prostate, larynx, hair follicle and colon epithelial cells (11,25). Bai et al (26) also reported maspin expression predominantly in the cytoplasm of trophoblastic cells. The various subcellular locations of maspin may be indicative of its numerous functions (11). To date, the most notable intracellular and extracellular biological functions of maspin have included promoting cell adhesion and apoptosis, and inhibiting cell motility, invasion and angiogenesis (11,25,27,28). In the current study, it was speculated that the cytoplasmic expression of maspin in normal or benign tissues and the nuclear expression of maspin in malignant tissues was associated with tumor inhibition and good prognosis.

Maspin expression levels decreased gradually from normal first-trimester placenta to rHMs, mHMs and IM/CCAs, whereas the expression of m-p53 increased. In addition, GTNs exhibited significantly lower expression levels of maspin and higher expression levels of m-p53 than rHMs. The results of the present study also indicated that the expression of maspin was inversely correlated with the expression of m-p53 in HMs, which was similar to the results reported for gastric cancer (29,30). Furthermore, the expression of maspin was inversely correlated, and that of m-p53 positively correlated, with a number of prognostic factors, including serum β-hCG levels, uterine size and diameter of theca-lutein cysts; however, age was not found to be associated. In GTNs, expression of maspin was associated with a lower FIGO prognostic score, whereas expression of m-p53 was associated with an advanced FIGO stage. Overall, HMs with negative expression of maspin and positive expression of m-p53 were strongly associated with poor prognosis and a high risk of developing GTNs.

In conclusion, although the current study was small and the data requires further validation, these results demonstrate for the first time that there is downregulation of maspin and upregulation of m-p53 expression in GTDs, particularly in those that develop GTN. The pathogenesis and prognostic roles...
of maspin and p53 in GTD are implied, and warrant further studies to reveal the underlying mechanisms and potential clinical applications.

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