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Promoter Hypermethylation of Multiple Genes in Hydatidiform Mole and Choriocarcinoma

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The methylation status of genes in hydatidiform mole and choriocarcinoma and its significance is relatively unexplored. We investigated the methylation status of the promoter regions of six genes, *p16*, *HIC-1*, *TIMP3*, *GSTP1*, death-associated protein kinase (*DAPK*), and *E-cadherin* in 54 hydatidiform moles, five choriocarcinomas, and 10 first trimester placenta by methylation-specific polymerase chain reaction (PCR). Immunohistochemical expression of p16, TIMP3, and E-cadherin, and quantitative real-time RT-PCR of p16 was also performed. Among the six genes examined, the promoter region of four genes (*E-cadherin*, *HIC-1*, *p16*, *TIMP3*) in choriocarcinoma and three genes (*E-cadherin*, *HIC-1*, *p16*) in hydatidiform mole exhibited aberrant methylation whereas none was hypermethylated in normal placenta. There was a significant correlation between methylation and reduced expression of p16, E-cadherin, and TIMP3 (P < 0.001). Fifteen of the 54 patients with hydatidiform mole developed gestational trophoblastic neoplasia requiring chemotherapy. Promoter hypermethylation of *p16* alone, or combined with *E-cadherin*, was significantly correlated to such development (P = 0.001, 0.0005, respectively). Hypermethylation of multiple genes, especially *p16*, might be related to the subsequent development of gestational trophoblastic neoplasia. *(J Mol Diagn 2004, 6:326–334)*

Hydatidiform mole and choriocarcinoma are two major subtypes of gestational trophoblastic disease (GTD) in Orientals. The reported incidence of GTD is approximately 1 in 500 to 1 in 1000 pregnancies in Asia, two to ten times higher than that in United States and Europe.[1,2] Choriocarcinoma is a highly malignant epithelial tumor arising from the trophoblast of any type of gestational event, most often a hydatidiform mole. Hydatidiform mole represents abnormally formed placenta characterized by swelling of chorionic villi and excessive proliferation of trophoblast. Although not a frank malignancy, hydatidiform mole may behave in an aggressive way with a predisposition for malignant transformation. About 8 to 30% of patients with hydatidiform mole will develop gestational trophoblastic neoplasia and require chemotherapy.[1,2] Most choriocarcinomas are related to previous hydatidiform moles. Similar to other human cancers, neoplastic transformation of trophoblast is likely to be a multistep process and involves multiple genetic alterations including activation of oncogenes and inactivation of tumor suppressor genes.[3,4] Potential biological markers related to malignant transformation of trophoblast include c-erbB-2,[5] cyclin E,[6] DOC-2/hDab2,[7] Ras GTPase activating protein,[8] telomerase activity,[9] apoptotic index,[10,11] and matrix metalloproteinases,[12] but no molecular parameter can currently replace serial serum human chorionic gonadotrophin (hCG) titers as the mainstay for predicting gestational trophoblastic neoplasia.

Promoter hypermethylation has recently been found to be an important epigenetic mechanism causing gene inactivation. Tumor suppressor genes, involving cell cycle regulation (*p16*), DNA repair and protection (*BRCA1* and *GSTP1*), apoptosis (*DAPK*), cell adherence and metastasis process (*E-cadherin*, *TIMP3*), may be silenced by promoter CpG island methylation in many human tumors, thus contribute to carcinogenesis. Previously, we have shown that hypermethylation of E-cadherin may be related to its reduced expression in hydatidiform mole using a relatively small number of cases.[13] While hypermethylation of the *H19* and *TFPI-2* genes has been reported in choriocarcinoma,[14–16] little is currently known about the methylation status of tumor suppressor genes in GTD.

In this study, we examined the frequency of methylation of six genes, *p16*, *E-cadherin*, *TIMP3*, *DAPK*, *GSTP1*, and *HIC-1*, which are known to undergo epigenetic inactivation in other human cancers, in 54 hydatidiform moles, five human gestational choriocarcinomas, and 10 normal first trimester placentas by methylation-specific polymerase chain reaction (MSP). Expressions of p16, TIMP3, and E-cadherin in these samples were investigated by

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Table 1. Primer Sequences and PCR Conditions for MSP

Gene*		Forward primers (5′→ 3′)	Reverse primer (5′→ 3′)	Annealing temperature (°C)	Product size (bp)
p16	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65	150
	U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	60	151
E-cadherin	M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	57	115
	U	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	53	97
TIMP3	M	CGTTTCGTTATTTTTTGTTTTCGGTTTTC	CCGAAAACCCCGCCTCG	59	116
	U	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCAAAAACCCCACCTCA	59	122
DAPK	M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	60	98
	U	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	60	106
GSTP1	M	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	59	91
	U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	59	97
HIC-1	M	TCGGTTTTCGCGTTTTGTTCGT	AACCGAAAACTATCAACCCTCG	64	95
	U	TTGGGTTTGGTTTTTGTGTTTTG	CACCCTAACACCACCCTAAC	62	118

*m, methylated sequence; u, unmethylated sequence.

immunohistochemistry and quantitative real-time RT-PCR. In addition, the relationship between the methylation status and subsequent development of gestational trophoblastic neoplasia was analyzed.

Materials and Methods

Patients and Samples

All specimens of trophoblastic tissue were collected at the Department of Pathology, University of Hong Kong, Queen Mary Hospital. Clinically and ultrasonographically suspected cases of hydatidiform moles were suction evacuated. First trimester samples were obtained after induced abortion by suction evacuation. Fresh chorionic villi, molar vesicles, and decidua were dissected, selected and snap-frozen in liquid nitrogen, and stored at −70°C. The rest of tissue was fixed in formalin. Frozen tissue blocks of 10 normal first trimester placentas (gestational age 9.0 ± 2.2 weeks) and 54 hydatidiform moles with follow-up data were selected for this study. The patients' age ranged from 16 to 51 (mean, 32.7) while the gestational age of the hydatidiform mole ranged from 6 weeks to 34 weeks (mean, 13.6 weeks). Archival formalin-fixed, paraffin-embedded tissue blocks from five choriocarcinomas before chemotherapy were also retrieved. The histological features of these cases were assessed using generally agreed and accepted diagnostic criteria.[1,2] Some of the hydatidiform moles had also been examined previously, by chromosome *in situ* hybridization and genotyping using DNA extracted from laser-capture-microdissected tissues.[17]

Thirty-nine of the 54 hydatidiform moles were complete moles while 15 were partial moles. Gestational trophoblastic neoplasia was diagnosed if there was a plateau in hCG level for 4 weeks or if there was a further increase in hCG for 3 consecutive weeks when pregnancy was excluded.[18] According to these criteria, 39 patients had spontaneous regression of the hydatidiform mole while the other 15 cases developed gestational trophoblastic neoplasia and received chemotherapy.

MSP

Genomic DNA extracted from the frozen tissues was modified by sodium bisulfite as previously described with minor modification.[19,20] Briefly, one μg genomic DNA was denatured by adding freshly prepared sodium hydroxide (final concentration of 0.3 mol/L) at 37°C for 10 minutes, and 95°C for 2 minutes. After cooling down to room temperature, hydroquinone (30 mmol/L) and sodium bisulfite (3.6 mol/L, pH 5.0) were added. The mixture was then incubated at 55°C for 15 to 16 hours in the dark. Wizard DNA Cleanup System (Promega, Madison, WI) was applied to purify the sodium bisulfite-modified DNA according to manufacturer's recommendations. The modified DNA was then incubated in 0.3 mol/L sodium hydroxide at 37°C for 15 minutes, then neutralized by adding sodium acetate (pH 4.0). The DNA was ethanol-precipitated and re-suspended in 30 μl of 10 mmol/L Tris water (pH 8.0). The primers and reaction conditions of MSP for the investigated genes were listed in Table 1. Five μl of each PCR products were separated on 2.5% agarose gel, which was stained with ethidium bromide, and directly visualized under UV illumination. DNA from normal placenta treated with SssI methylase (New England Biolabs, Beverly, MA) was used as a methylation-positive control. Genomic DNAs without bisulfite modification were used as negative control in both methylated and unmethylated reactions. Samples demonstrated to have methylation in either gene were repeated for confirmation.

Immunohistochemistry

Sections 5-μm thick were cut from representative paraffin blocks of each case and mounted on 2% aminopropyltriethoxysilane-coated glass slides. To avoid antigen loss after preparation, immunostaining was carried out within 48 hours. After microwave pretreatment for antigen retrieval, primary antibodies for E-cadherin (monoclonal, at a dilution 1:150; Transduction Laboratories, Lexington, KY), p16 (monoclonal, at a dilution 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), TIMP3 (monoclo-

nal, at a dilution 1:100; Oncogene Research Products, Boston, MA) were added and incubated overnight at 4°C. Biotin-labeled anti-mouse immunoglobulin (Ig) G (Dako, Glostrup, Denmark) was used as secondary antibody. Diaminobenzidine-hydrogen peroxide was used as chromogen. A light Mayer's hematoxylin counter-stain was used. Negative controls were prepared by replacing the primary antibody with Tris-buffered saline. According to a previous report, we defined the expression status as normal or reduced if ≥80%, or <80% of trophoblast cells displayed positive staining, respectively.[21]

Quantitative Real-Time RT-PCR

Twelve hydatidiform moles, six subsequently regressed, and six developed gestational trophoblastic neoplasia, were selected for quantitative RT-PCR analysis according to previously published procedures.[22] First-strand cDNA was synthesized from DNase-treated total RNA with oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), followed by PCR amplification using the corresponding gene-specific primers. Primer sequences for p16 are as follows: 5′- TAC GGT CGG AGG CCG ATC CAG GTC-3′ (sense) and 5′- GGG GAT GTC TGA GGG ACC TTC CGC -3′ (antisense). GAPDH (housekeeping gene used as control in RT-PCR study): 5′-CTC AGA CAC CAT GGG GAA-3′ (sense) and 5′-ATG ATC TTG AGG CTG TTG-3′ (antisense). Quantitative real-time RT-PCR was performed in a 25-μl reaction, which included 1 μl of cDNA template, 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, pH 9.0, 0.05 mmol/L of each dNTP, 0.2 mmol/L of each forward and reverse oligo primer, 2.5 U of *Taq*DNA polymerase (Amersham Biosciences, Piscataway, NJ), and 0.1X SYBR Green I (Molecular Probes, Eugene, OR), using an iCycler iQ Multi Color Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Each PCR reaction was optimized to ensure that a single PCR product was amplified and no product corresponding to primer-dimer pairs was present. PCR reactions of each template were performed in duplicate in one 96-well plate. The PCR cycling conditions were as follows: 95°C for 3 minutes followed by 50 cycles of three steps at 95°C for 30 seconds, 68°C for 30 seconds, and then 72°C for 30 seconds. The relative fold change method was used to determine the relative quantitative gene expression for p16 compared with the GAPDH.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science 10.1 for Windows (SPSS Inc., Chicago, IL). Differences between groups were analyzed by the χ^2 test or Fisher's exact test where applicable. Quantitative real time RT-PCR data were analyzed by Wilcoxon test. Regression analysis was performed to test the correlation between the methylation status and the clinical parameter. *P* values less than 0.05 was considered statistically significant. All statistical tests were calculated in two-sided.

Results

MSP

We have analyzed the methylation status of the promoter region of six genes (*p16*, *E-cadherin*, *TIMP3*, *DAPK*, *GSTP1*, and *HIC-1*) in choriocarcinoma, hydatidiform mole, and normal placenta. In normal placenta, only unmethylated alleles could be detected for all of the six investigated genes.

The results of MSP in choriocarcinoma and hydatidiform mole were shown in Table 2. Representative examples of the MSP analysis were shown in Figure 1. At least one gene was methylated in 64% (38 of 59) of the GTD samples including 61% (33 of 54) of hydatidiform mole and 100% (5 of 5) of choriocarcinoma. Four genes showed aberrant methylation in choriocarcinoma at the frequencies of 40% (2 of 5) for *E-cadherin*, *p16*, *TIMP3*, and 60% (3 of 5) for *HIC-1*. Three genes showed aberrant methylation in hydatidiform mole at frequencies of 13.0% (7 of 54) for *E-cadherin*, 20.4% (11 of 54) for *p16* and 40.7% (22 of 54) for *HIC-1*. No significant correlation was found between methylation status of *E-cadherin*, *p16*, and *HIC-1* ($P > 0.05$).

The findings regarding correlation between methylation status of these genes, in isolation or combination, with subsequent development of gestational trophoblastic neoplasia was tabulated in Table 3. The adjusted *P* value for multiple testing was estimated by the Bonferroni procedure $\alpha' = 1 - (1 - 0.05)^{1/7} = 0.007$.

Those hydatidiform moles, which subsequently developed gestational trophoblastic neoplasia, were found to have significantly more frequent *p16* promoter hypermethylation comparing to those that subsequently regressed ($P = 0.001$, Fisher's exact test, OR = 13.714, 95% CI = 2.898 to 62.899). The sensitivity and specificity of *p16* hypermethylation in predicting subsequent development of gestational trophoblastic neoplasia were 53.5% (8 of 15) and 92.3% (36 of 39), respectively. Methylation status of *E-cadherin* and *HIC-1* by themselves showed no such correlation ($P = 0.084$ and 0.492, respectively). However, when the *E-cadherin* and *p16* methylation status were analyzed together, statistically significant correlation between promoter hypermethylation of either or both genes and development of gestational trophoblastic neoplasia was demonstrated ($P < 0.0005$, Fisher's exact test, OR = 18.7, 95% CI = 4.256 to 82.161). Moreover, the sensitivity and specificity in predicting subsequent development of gestational trophoblastic neoplasia became 73.5% (11 of 15) and 87.2% (34 of 39), respectively. On the other hand, combined analysis on the *E-cadherin*, *p16*, and *HIC-1* methylation status showed no significant correlation to the development of gestational trophoblastic neoplasia ($P = 0.037$, more than adjusted *P*–value 0.013, Fisher's exact test) with sensitivity and specificity of 80% (12 of 15) and 51.3% (20 of 39), respectively.

Regression analysis showed that *E-cadherin*, *HIC-1*, and *p16* promoter methylation neither correlated with patients'

Table 2. Methylation Profiles of the Investigated Genes in Hydatidiform Moles and Choriocarcinomas

Code	Diagnosis	Patient's age (years)	Gestational age (weeks)	E-cadherin	HIC-1	p16	TIMP3	DAPK	GSTP1
M3	CM, GTN	31	22			■			
M4	CM	23	9						
M5	CM	28	11						
M6	PM	44	6						
M12	PM, GTN	38	28						
M14	CM, GTN	24	16		■				
M16	PM	45	14	■	■	■			
M17	CM	25	10		■	■			
M20	CM	31	9	■	■				
M24	CM	35	11						
M25	PM	32	NA						
M26	CM	32	10		■				
M27	CM	34	12						
M28	CM, GTN	35	12						
M30	PM, GTN	30	NA			■			
M31	CM, GTN	27	11			■			
M32	CM, GTN	45	16	■	■				
M33	CM	51	16						
M34	CM	24	NA						
M35	CM	21	NA						
M42	CM	45	NA		■				
M48	CM	28	NA						
M49	PM	31	15						
M50	CM, GTN	35	18	■	■				
M51	CM	40	NA		■				
M52	CM	16	7						
M54	CM	48	NA						
M55	CM	24	17						
M56	PM	29	NA		■				
M57	CM, GTN	30	NA	■		■			
M61	PM	37	14	■					
M62	CM	33	NA						
M63	CM	25	10		■				
M64	CM, GTN	44	NA		■	■			
M66	CM, GTN	43	NA		■	■			
M67	CM, GTN	37	11			■			
M70	CM	34	NA						
M71	CM	25	18						
M72	CM	19	13		■				
M73	CM	18	NA						
M74	CM	29	NA		■				
M77	CM, GTN	38	12	■					
M78	PM	39	34		■				
M79	PM	32	7		■				
M80	PM	29	9		■				
M81	CM	23	19						
M82	CM	30	NA		■				
M89	PM	42	6			■			
M91	CM, GTN	24	9			■			
M92	CM	33	13		■				
M95	PM	50	12		■				
M99	PM	35	NA						
M100	CM	31	NA		■				
M102	PM, GTN	35	19						
C1	CCA	33	NA	■	■	■			
C2	CCA	42	NA	■	■				
C3	CCA	39	NA				■		
C4	CCA	28	NA			■			
C5	CCA	37	NA		■		■		

Filled boxes, methylated results; open boxes, unmethylated results; CM, complete mole; PM, partial mole; GTN, subsequent development of gestational trophoblastic neoplasia; NA, not available.

age (P = 0.12, 0.24, and 0.46, the adjusted R^2 = 0.028, 0.0080, and 0, respectively) nor with the gestational age of hydatidiform moles (P = 0.92, 0.97, and 0.39, respectively, the adjusted R^2 = 0) in our studied samples.

Methylated alleles of *DAPK* and *GSTP1* cannot be detected in either choriocarcinoma or hydatidiform mole. Aberrant methylation of *TIMP3* was detectable in choriocarcinoma but not in hydatidiform mole.

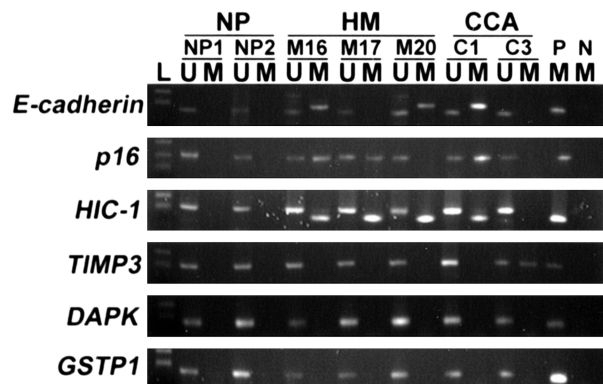

Figure 1. Representative examples of MSP findings in normal placenta (NP), hydatidiform mole (HM), and choriocarcinoma (CCA). The PCR products in the **lanes** marked U showed the presence of unmethylated alleles of each gene, whereas the products in the **lanes** marked M indicate the presence of methylated alleles. L, size marker (pBSK/MSP I DNA marker); P, positive control; N, negative control.

Immunohistochemical Analysis

To determine whether the observed aberrant methylation was associated with gene silencing, we examined the expression of p16, E-cadherin, and TIMP3 in 54 hydatidiform moles and five choriocarcinomas using immunohistochemistry. P16 immunoreactivity was mainly demonstrated in the nuclei of the trophoblasts while TIMP3 and E-cadherin expression was predominantly found at the cytoplasm and cell membrane. Reduced expression was defined if less than 80% trophoblastic cells showed positive staining.[21] Table 4 and Figure 2 showed the findings for the immunohistochemical staining of these three proteins and their relationship with the MSP results. A close correlation was noted between hypermethylation of *p16*, *E-cadherin*, and *TIMP3* and reduced expression of the corresponding protein ($P < 0.001$).

Quantitative Real-Time RT-PCR

Six cases of hydatidiform mole which had subsequently developed gestational trophoblastic neoplasia, and six cases which had subsequently regressed, were further analyzed by quantitative real-time RT-PCR to explore the RNA expression of p16. The expression levels of p16 in these 12 cases were shown in Figure 3 and concomitant to the immunostaining results. The mRNA level of p16 in methylated cases was significantly less than in those without methylation ($P < 0.01$). Moreover, in hydatidiform mole cases that subsequently developed gestational trophoblastic neoplasia, the expression of p16 was significantly lower than in hydatidiform moles with spontaneous remission ($P = 0.026$).

Discussion

Accumulating evidence suggests that changes in gene expression through epigenetic mechanism play important roles for tumor progression. Hydatidiform mole is associated with an increased risk of subsequent development of gestational trophoblastic neoplasia and choriocarcinoma but the mechanism by which such aggressive progression is mediated remains unknown. In this study, a relatively large collection of GTD samples and normal placentas was analyzed for methylation status of six genes which have been found to be frequently meth-

Table 3. Correlation of Methylation Status in Hydatidiform Moles with the Development of Gestational Trophoblastic Neoplasia (GTN)

Gene	Status	Regressive moles Freq	Regressive moles (%)	GTN Freq	GTN (%)	P-value (Fisher or *Chi-square)	OR, 95% CI
E–cad HIC-1 p16	U	20	51.3%	3	20.0%		
	M	19	48.7%	12	80.0%	0.037*	4.211, 1.026 to 17.288
	Total	39		15			
E–cad p16	U	34	87.2%	4	26.7%		
	M	5	12.8%	11	73.3%	<0.0005	18.7, 4.256 to 82.161
	Total	39		15			
E–cad HIC-1	U	21	53.8%	8	53.3%		
	M	18	46.2%	7	46.7%	0.973*	
	Total	39		15			
HIC1 p16	U	21	53.8%	4	26.7%		
	M	18	46.2%	11	73.3%	0.073	3.208, 0.869 to 11.845
	Total	39		15			
E–cad	U	36	92.3%	11	73.3%		
	M	3	7.7%	4	26.7%	0.084	
	Total	39		15			
HIC-1	U	22	56.4%	10	66.7%		
	M	17	43.6%	5	33.3%	0.492*	
	Total	39		15			
p16	U	36	92.3%	7	46.7%		
	M	3	7.7%	8	53.3%	0.001	13.714, 2.898 to 62.899
	Total	39		15			

Table 4. Correlation of MSP Results with Protein Expression in Hydatidiform Moles and Choriocarcinomas

Protein	Expression status	MSP results		P–value
		M	U	
E-cadherin	Normal	0	44	<0.001
	Reduced	9	6	
p16	Normal	2	42	<0.001
	Reduced	11	4	
TIMP3	Normal	0	57	<0.001
	Reduced	2	0	

ylated in other human cancers.[21,23] At least one gene was hypermethylated in two-thirds of the hydatidiform mole samples and up to 100% of choriocarcinoma samples. In contrast, no aberrant methylation was detected in 10 normal first trimester placentas at all of the tested genes. This indicated that aberrant CpG island methylation is a frequent and probably disease-restricted event in GTD, including both hydatidiform mole and choriocarcinoma, though more common in choriocarcinoma.

In particular, our data suggested that inactivation of the *p16* gene through methylation was more likely to be associated with the malignant transformation of trophoblast. As observed in this study, hypermethylation of *p16* occurred more frequently among patients with gestational trophoblastic neoplasia or choriocarcinoma than those with regressed disease.

We also demonstrated that *p16* promoter hypermethylation by itself was relatively specific in predicting gestational trophoblastic neoplasia development while combined analysis of *p16* and *E-cadherin* methylation status was more sensitive in such prediction. While methylation status of *E-cadherin* alone showed no statistically significant correlation with clinical outcome, it improves the sensitivity of predicting gestational trophoblastic neoplasia when analyzed together with *p16* methylation status. The methylation of *E-cadherin* may thus still play a role in the progress of hydatidiform mole. Further studies on may be able to elucidate the mechanisms. On the other hand, combined analysis on the *E-cadherin*, *p16*, and *HIC-1* methylation status showed no significant correlation to the development of gestational trophoblastic neoplasia. *HIC*-1 methylation status may not play a role in such disease progress.

p16 encodes a protein belonging to the cyclin-dependent-kinase-inhibit family, inhibits Cdk4/6 and cyclin D association, thus prevents the cell entering the S phase.[24]

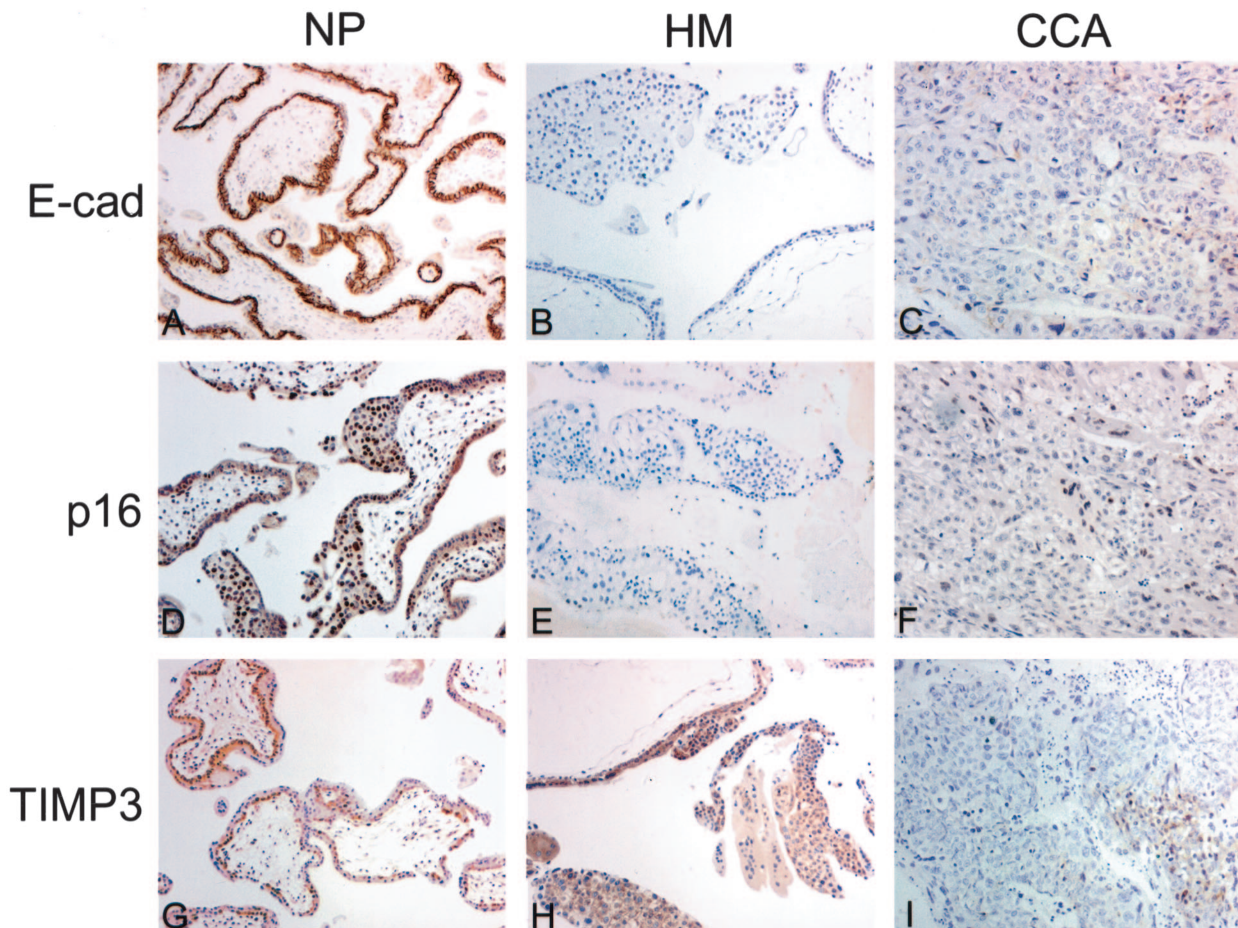

Figure 2. Immunostaining of E-cadherin, p16, and TIMP3 in normal placenta (NP), hydatidiform mole (HM), and choriocarcinoma (CCA). All three proteins were strongly expressed in normal placentas, but significantly reduced in choriocarcinomas showing hypermethylation. In hydatidiform moles showing hypermethylation of *E-cadherin* (M50) or *p16* (M31), expression of corresponding proteins was reduced. Expression of TIMP3 was strong in hydatidiform mole.

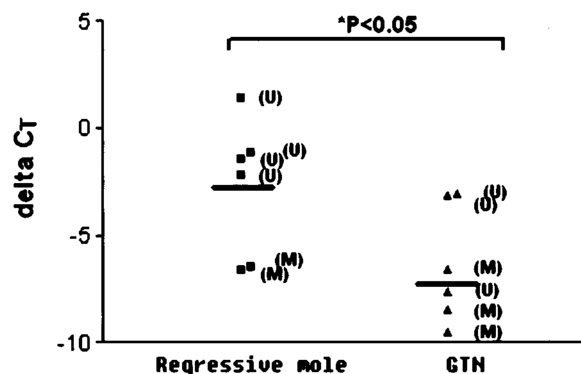

Figure 3. Quantitative real-time RT-PCR analysis of p16 expression in regressive mole and those developed gestational trophoblastic neoplasia (GTN) as illustrated by the ratio of signals from p16 and GAPDH housekeeping gene, measured by threshold cycle value (ΔC_T). Expression of p16 was significantly lower in GTN than in regressive mole.

Aberrant methylation of *p16* associated with a loss of expression in tumor cells was first reported by Herman et al[20] This phenomenon may be analogous to homozygous deletion, leading to reduced *p16* expression and a selective growth advantage for tumor cells. Methylation silencing of *p16* was found to be frequent in some types of tumors, including colon, prostate, esophageal, and pancreatic carcinomas.[25–28] In normal placenta, *p16* remains unmethylated, as confirmed in the present report.[29] In GTD, our findings corroborated previous immunohistochemical study in which reduction of *p16* expression was observed in malignant trophoblastic tumors.[30] However, this is the first report evaluating the methylation status of *p16* and its clinical significance. In this study, we documented a statistically significant correlation between methylation status and expression level of *p16* in GTD.

Little is known about the molecular events involved in the pathogenesis of choriocarcinoma. Genes involved in the control of metastatic potential in human cancers, such as *nm23*, *Kiss-1*, and *E-cadherin* have been reported to be down-regulated in choriocarcinoma.[5,31,32,13] Recently, reduced expression of TFPI-2 due to promoter hypermethylation was observed in choriocarcinoma cells.[15] Hypermethylation of *TIMP3*, which was detected in choriocarcinoma but not in hydatidiform mole in our current study, might be a late event involved in the development and progression of choriocarcinoma. Interestingly, both TFPI-2 and TIMP3 effectively decreased the activation of metalloproteinases, thus inhibiting degradation of the extracellular matrix and reducing the invasive potential of tumor cells.[15,33] In addition, TIMP3 also has some unique properties in human cancer development, such as growth suppression,[34] inhibition of angiogenesis,[35,36] and induction of apoptosis.[37,38] Transcriptional inactivation of *TIMP3* by hypermethylation has been found to associated with a more malignant and invasive phenotype in some human cancer cells.[21,39,40] Therefore, down-regulation of TIMP3 in choriocarcinoma might be involved in inactivation of several tumor suppressive mechanisms. The accurate roles of *TIMP3* in malignant trophoblast remained to be uncovered by further study.

The *HIC-1* gene, located on chromosome 17p13.3, is unusual in that the entire gene is contained in a CpG-rich region. Previous studies showed that *HIC-1* was commonly hypermethylated and transcriptionally inactivated in leukemia, medulloblastoma, and cancers of breast, prostate, and uterine cervix.[23,41–44] In the present study, *HIC-1* was the most frequently methylated gene among the panel of genes studied in both hydatidiform moles as well as in choriocarcinoma. This indicated that methylation of *HIC-1* might be an early event involved in the pathogenesis of GTD.

Regarding *DAPK* and *GSTP1*, we could not detect hypermethylation in either gene in the GTD samples examined. Although these genes were frequently methylated in other tumor types, they were apparently not the targets for methylation in GTD. Similar organ-specific methylation patterns have been reported.[21,45,46]

Recent improvements in the quality of sonography have resulted in the earlier detection of abnormal pregnancy and earlier evacuation, thus increasing the chances of encountering difficulties in histological distinction between hydatidiform mole and a non-molar abortion exhibiting hydropic change and trophoblast hyperplasia.[4] Given the relative high rate of methylation of *HIC-1* and to a lesser extent, *p16* and *E-cadherin* in hydatidiform mole, detection of aberrant methylation of these genes by MSP might be an adjunct tool in differentiating molar and non-molar pregnancy.

The careful follow-up of patients with hydatidiform mole has undoubtedly contributed to the dramatic reduction in mortality from choriocarcinoma. However, such follow-up involves frequent serial clinic visits and β-hCG assay, creating burden on both the patients and the community resources. In reality, less than 30% of hydatidiform mole patients need such an intensive care. In addition, much debate exists on the timing regarding initiation of chemotherapy for women at risk for gestational trophoblastic neoplasia. If the patients who will develop gestational trophoblastic neoplasia could be identified at the time of the original diagnosis, there would be a significant benefit in reduction of unnecessary follow-up. The outcome of hydatidiform mole can be evaluated using MSP and immunohistochemical studies on candidate genes immediately after evacuation and the selection of high-risk patients and initiation of prophylactic chemotherapy can thus be guided. Our data suggested that *p16* may be a candidate deserving further study.

In conclusion, using candidate genes approach, we found that hypermethylation of tumor suppressor genes frequently occurred in choriocarcinoma and hydatidiform mole. This report provides preliminary evidence that hypermethylation of *p16* in relation to its decreased expression might serve as a potential marker predicting subsequent development of gestational trophoblastic neoplasia. However, more conclusive interpretation of our observation needs to be confirmed in further studies involving larger sample size and preferably quantitative methylation study.

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