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mRNA Expression Profiling in Hydatidiform Mole: Comparison between Pre-Gestational Trophoblastic Neoplasia Moles and Remission Moles

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ABSTRACT

Objectives: To explore potential mRNAs that can predict malignant transformation and identify mRNAs expression profile in complete hydatidiform mole.

Materials and Methods: A case-control study was conducted between complete hydatidiform moles that turned out to be postmolar gestational trophoblastic neoplasia (postmolar GTN) and complete hydatidiform moles that regressed spontaneously after evacuation (remission mole). We quantitatively assessed the expression of 770 human cancer genes from formalin-fixed paraffin embedded (FFPE) specimens or fresh frozen tissues using Nanostring nCounter. The differentially expressed genes between postmolar GTN group and remission mole group were analyzed.

Results: There were 12 cases recruited in this study: 6 remission moles and 6 postmolar GTN. Seven hundred and seventy genes were analyzed showing 29 genes that were significantly different in GTN moles compared to the remission moles. Nine of these genes (*JUN, COL4A6, SOCS3, PLA2G10, NFKBIZ, FGFR3, CACNA1D, FGF7 and PLAU*) were significantly different for more than 2 folds. The JUN had the highest different ratio and the lowest p value when compared between 2 groups (3.26 folds, p = 0.003). After reviewing their functions, JUN plays a role in several cancer initiations. These genes are promising biomarkers for prediction of postmolar GTN.

Conclusion: The analysis of mRNA profiles can distinguish between complete hydatidiform moles that will remission from those that will turn out to postmolar GTN. We identified 29 genes that were differentially expressed between the two groups. The results lead to further investigations on candidate genes and could probably explain the mechanism of malignant transformation to postmolar GTN.

Keywords: gestational trophoblastic neoplasia, postmolar GTN, mRNA, nanostring.

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เปรียบเทียบการแสดงออกของ mRNA ในครรภ์ไข่ปลาอุกชนิดที่กลายเป็นมะเร็งครรภ์ไข่ปลาอุกและชนิดที่หายขาด

วิลาสินี อารีรักษ์, พฐู ตัณฑ์ไพโรจน์, ชนพ ช่วงโชติ, ชินโชติ ธีรภัคภิญโญ, เรืองศักดิ์ เลิศชจรสุข

บทคัดย่อ

วัตถุประสงค์*: เพื่อศึกษาหา mRNA ที่มีแนวโน้มจะใช้พยากรณ์การกลายเป็นมะเร็งครรภ์ไข่ปลาอุก และศึกษาการแสดงออกของ mRNA ในครรภ์ไข่ปลาอุก*

วัสดุและวิธีการ*: การศึกษาแบบมีกลุ่มควบคุมเปรียบเทียบระหว่างครรภ์ไข่ปลาอุกชนิดที่กลายเป็นมะเร็งครรภ์ไข่ปลาอุกและชนิดที่หายขาด ผู้เข้าร่วมการศึกษาจะได้รับการตรวจวัดปริมาณการแสดงออกของ mRNA 770 ชนิด ที่พบในมนุษย์ โดยตรวจจากชิ้นเนื้อจากพาราฟินบล็อก หรือชิ้นเนื้อแช่แข็ง ด้วยวิธี Nanostring nCounter จากนั้นทำการแปลผลของ mRNA ที่มีความแตกต่างกันระหว่างทั้งสองกลุ่ม*

ผลการศึกษา*: ผู้เข้าร่วมการศึกษาทั้งหมด 12 ราย แบ่งเป็นครรภ์ไข่ปลาอุกชนิดที่หายขาด 6 ราย และชนิดที่กลายเป็นมะเร็ง 6 ราย จาก mRNA 770 ชนิดที่ตรวจวัดปริมาณ พบว่ามี 29 ชนิดที่มีระดับการแสดงออกแตกต่างกันอย่างมีนัยสำคัญทางสถิติ โดย 9 ชนิดมีระดับการแสดงออกแตกต่างกันเกิน 2 เท่า ได้แก่ JUN, COL4A6, SOCS3, PLA2G10, NFKBIZ, FGFR3, CACNA1D, FGF7 และ PLAU ยีนที่มีการแสดงออกแตกต่างระหว่างสองกลุ่มมากที่สุด ได้แก่ JUN ซึ่งมีการแสดงออกในกลุ่มที่หายขาดเป็น 3.26 เท่าของกลุ่มที่กลายเป็นมะเร็ง (p = 0.003) โดยพบว่า JUN มีบทบาทสำคัญในการเกิดของมะเร็งหลายชนิด mRNA เหล่านี้จึงมีความน่าสนใจในแง่ของแนวโน้มที่จะใช้พยากรณ์การกลายเป็นมะเร็งครรภ์ไข่ปลาอุก*

สรุป*: การแสดงออกของ mRNA มีความแตกต่างกันระหว่างครรภ์ไข่ปลาอุกชนิดที่กลายเป็นมะเร็งครรภ์ไข่ปลาอุกและชนิดที่หายขาด จากการศึกษานี้พบว่ามี 29 ชนิดที่มีระดับการแสดงออกแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ผลจากการศึกษานี้จะสามารถนำไปสู่การศึกษาเพิ่มเติมเฉพาะใน mRNA บางชนิด ซึ่งอาจช่วยอธิบายการกลายเป็นมะเร็งของครรภ์ไข่ปลาอุก*

คำสำคัญ*: ครรภ์ไข่ปลาอุก, มะเร็งครรภ์ไข่ปลาอุก, mRNA, nanostring*

Introduction

Gestational trophoblastic disease (GTD) is a pregnancy-related disorder originating from abnormal proliferation of the placental trophoblasts. It can be classified as a benign disorder or hydatidiform mole and a malignant disorder or gestational trophoblastic neoplasia (GTN), of which the latter includes the invasive mole, choriocarcinoma, placental-site trophoblastic tumor and epithelioid trophoblastic tumor[1, 2]. Generally, hydatidiform moles would regress after evacuation, but about 15% of complete hydatidiform moles (CHMs) and 0.5-1% of partial hydatidiform moles (PHMs) could progress into postmolar GTN[2]. The risk of developing postmolar GTN varies among different regions in the world. In Thailand, there is a higher rate of developing postmolar GTN which was approximately 26%[3].

In order to detect postmolar GTN, several human chorionic gonadotropin (hCG) regression models have been used[4]. However, identifying patients who would have postmolar GTN at the time of evacuation would be of greater value because intensive follow-up monitoring can be planned, and in some cases, prophylactic chemotherapy can be given to prevent malignant transformation[5].

Several molecular markers have been studied in GTD. Tumor protein p53 (p53), p21, retinoblastoma gene (Rb) and mouse double minute 2 homolog (mdm^2) showed stronger expression in complete hydatidiform mole and choriocarcinoma than in partial mole[6]. There are few studies of panel genes expression in GTD. Kato et al[7] used microarray analysis to investigate the expression profiles of 589 genes committed to cell growth control in order to characterize the regulatory circuitry for cell proliferation in complete moles. A total of 57 genes were significantly upregulated in complete moles. These involved the Ras-mitogen activated protein kinase III (Ras-MAPKIII), janus kinase-signal transducer and activator of transcription 5 (JAK-STAT5) and Wnt signal pathways, implicating growth factor or cytokine-mediated signal pathways in the trophoblastic hyperplasia of complete moles. Several genes associated with anti-apoptosis, cell structuring and/or cell attachment were also upregulated in complete moles. In contrast, relatively fewer genes were downregulated and these involved insulin growth factor binding proteins (IGFBPs), versican, interleukin-1, tumor necrosis factor receptor, CD44 and Rad 52.

There is limited data on gene profiling in GTD, especially comparison between non cancer hydatidiform moles (remission moles) and moles that turn to be GTN (GTN moles). Therefore, we assessed the messenger ribonucleic acids (mRNAs) that were differentially expressed between CHMs that turned out to be GTN (GTN moles) and CHMs that regressed spontaneously after evacuation (remission moles), and also studied the profiles of mRNA expression in CHMs.

Materials and Methods

This study was a case-control study conducted at the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Ethical approval was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. The medical records of all patients with pathologically confirmed CHMs diagnosed between January 2007 and December 2016 were reviewed. Cases of CHMs with formalin-fixed paraffin embedded (FFPE) or fresh frozen specimens kept at the division of Gynecologic Pathology at King Chulalongkorn Memorial Hospital who completed clinical follow-up periods of at least one year were included in the study. After the GTN moles were selected, the remission moles were matched to the GTN moles using the same year of specimen retrieval with the nearest gestational age at diagnosis. All specimens were pathologically reassessed by two pathologists. Cases with low quality specimens or inadequate tumor tissues for

RNA extraction were excluded from the study.

RNA extraction

Total RNA was extracted from formalin fixed, paraffin embedded (FFPE) or fresh frozen tissue using RNeasy FFPE kit according to the manufacturer's instruction (Qiagen, Germantown, MD) and performed at Chula GenePRO Center, Faculty of Medicine, Chulalongkorn University. The lesions of interest (villous tissues) were reviewed and selected by the pathologist. Three to five ribbons of 10-µm FFPE tissue section were used for total RNA extraction. Then, RNA concentration and quality were determined by using nanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was stored at -80^{O}C until mRNA expressions were analyzed.

mRNA expression analysis

Analysis of mRNA expression was performed using nCounter Analysis System and PanCancer Human Pathways Assay Kit according to the manufacturer's instruction (nanoString®, Seattle, WA). One hundred nanograms of each total RNA sample were mixed with the probes and incubated at 65^{O}C for 16 hours. Then probe-hybridized samples were processed on the nanoString prep station. The processed cartridges were then transferred to the nanoString® digital analyzer and scanned on HIGH mode for 280 fields of view per sample. The nSloverTM Analysis software 3.0 (nanoString Technologies, Seattle, WA) was used to perform background subtraction, spike-in-control normalization and reference genes normalization.

Twelve FFPE specimens of CHMs (6 remission moles and 6 GTN moles) were done to discover the differentially expressed mRNA candidates between remission moles and GTN moles. Sample size calculation was based upon the mean difference and standard deviation of the count of mRNA candidate discovered from this study. Statistical analysis was performed using SPSS version 22 (SPSS Inc, Chicago, IL, USA). Baseline clinical data were presented as mean, median and percentage. Level of expression of each mRNA was presented as median count. The differentially expressed mRNAs between GTN moles and remission moles were analyzed using the Mann-Whitney U test. A p value of < 0.05 was considered as statistically significant.

Results

In this study, 6 remission moles and 6 GTN moles were analyzed. Baseline clinical data are shown in Table 1. The age and gestational age at diagnosis of molar pregnancy in both groups were similar. Pretreatment βhCG was higher in the GTN moles group [525,020 mIU/ml (238,759.75, 1,000,000) vs 179,973.5 mIU/ml (150,330.25, 217,952.75)]. The patients in both groups were treated mostly with suction curettage (66.7%).

Among 770 human mRNAs evaluated, 29 mRNAs were found to be differentially expressed between the two groups ($p < 0.05$). Table 2 shows the median count of each of the differentially expressed 29 mRNAs found in this study.

The largest differences (more than 2 folds downregulations) were seen in 9 mRNAs: JUN, COL4A6, SOCS3, PLA2G10, NFKBIZ, FGFR3, CACNA1D, FGF7 and PLAU. Clustering analysis of these 9 mRNAs demonstrated a tree with obvious distinction between remission moles and GTN moles. (Fig. 1.).

Among these 9 mRNAs, JUN demonstrated the largest difference with a median count of 35.62 in GTN moles vs 115.96 in remission moles ($p = 0.003$). COL4A6 had 3.18 folds different in median count between GTN moles and remission moles ($p = 0.041$), and SOCS3 had 3.09 folds different in median count between GTN moles and remission moles ($p = 0.012$).

This study used 10 samples of FFPE specimen and 2 samples of fresh specimen. The amount of mRNAs count was not different between these 2 sources of specimens.

Table 1. Demographic data.

	GTN (n = 6)	Remission (n = 6)
Age (years)	27 (19, 47.5)	31.5 (24.75, 44.75)
GA (weeks)	10 (7.5, 14.5)	12.5 (10.5, 16)
Pretreatment βhCG (mIU/ml)	525,020 (238,759.75, 1,000,000)	179,973.5 (150,330.25, 217,952.75)
Treatment		
• Suction curettage	4 (66.7%)	4 (66.7%)
• Hysterectomy	2 (33.3%)	2 (33.3%)
Duration to normal βhCG (days)		70.5 (51.25, 87.0)
Duration to GTN (days)	49 (19.25, 55.5)	
hCG at Dx GTN (mIU/ml)	7469 (1380, 118553.5)	
Stage		
Stage I	3 (50%)	
Stage II	0 (0%)	
Stage III	2 (33.3%)	
Stage IV	0 (0%)	
Missing	1 (16.7%)	
Risk score		
Low risk	5 (83.3%)	
High risk	0 (0%)	
Missing	1 (16.7%)	
Chemotherapy regimen		
Methotrexate	3 (50%)	
Actinomycin D	1 (16.7%)	
None (Surgical treatment – hysterectomy)	1 (16.7%)	
Missing	1 (16.7%)	

Data are presented as median (interquartile range) and number (percentage)

GTN: gestational trophoblastic neoplasia, GA: gestational age, hCG: human chorionic gonadotropin, Dx: diagnosis

Table 2. Significantly different expressed mRNAs between gestational trophoblastic neoplasia moles group and the remission moles group ($p < 0.05$).

mRNA	GTN (n = 6)	Remission (n = 6)	Fold change	p value
JUN	35.62	115.96	-3.26	0.003
COL4A6	5.71	18.17	-3.18	0.041
SOCS3	89.13	275.76	-3.09	0.012
PLA2G10	18.72	6.22	3.01	0.033
NFKBIZ	52.54	131.7	-2.51	0.009
FGFR3	100.73	43.91	2.29	0.020
CACNA1D	3.18	7.3	-2.29	0.025
FGF7	5.57	12.63	-2.27	0.033
PLAU	32.09	71.8	-2.24	0.023
CCNB1	126.75	64.34	1.97	0.046
MYC	37.25	72.69	-1.95	0.028
SUV39H2	61.71	31.84	1.94	0.006
CACNA1G	8.99	16.97	-1.89	0.021
SFRP4	3.85	7.24	-1.88	0.034
PRKACB	4.83	8.85	-1.83	0.039
WNT3	9.07	16.64	-1.83	0.044
LEPR	18.47	31.76	-1.72	0.028
LAMC3	20.06	33.35	-1.66	0.009
BMP4	17.38	28.73	-1.65	0.031
NFKBIA	203.76	321.85	-1.58	0.049
NFE2L2	40.96	64	-1.56	0.019
DUSP6	37.79	58.35	-1.54	0.042
GNG12	260.05	171.16	1.52	0.047
SOCS2	49.86	75.05	-1.51	0.045
ABL1	43.86	63.94	-1.46	0.015
CCND3	57.82	83.63	-1.45	0.048
SOS1	53.66	75.69	-1.41	0.023
CREB3L1	92.74	127.79	-1.38	0.047
ARID1B	74.03	98.66	-1.33	0.042

Data are presented as median

JUN: Jun proto-oncogene, COL4A6: collagen type IV alpha-6, SOCS3: suppressor of cytokine signaling 3, PLA2G10: phospholipase A2 group X, NFKBIZ: nuclear factor kappa-B inhibitor zeta, FGFR3: fibroblast growth factor receptor 3, CACNA1D: calcium voltage-gated channel subunit alpha-1D, FGF7: fibroblast growth factor 7, PLAU: plasminogen activator urokinase, CCNB1: cyclin B1, MYC: MYC proto-oncogene, SUV39H2: suppressor of variegation 3-9 drosophila homolog of 2, CACNA1G: calcium voltage-gated channel subunit alpha-1G, SFRP4: secreted frizzled-related protein 4, PRKACB: protein kinase cAMP-activated catalytic subunit beta, WNT3: wingless-type MMTV integration site family member 3, LEPR: leptin receptor, LAMC3: laminin gamma-3, BMP4: bone morphogenetic protein 4, NFKBIA: nuclear factor kappa-B inhibitor alpha, NFE2L2: nuclear factor erythroid 2-like 2, DUSP6: dual-specificity phosphatase 6, GNG12: guanine nucleotide-binding protein gamma 12, SOCS2: suppressor of cytokine signaling 2, ABL1: ABL proto-oncogene 1, CCND3: cyclin D3, SOS1: SOS Ras/Rac guanine nucleotide exchange factor 1, CREB3L1: cAMP responsive element binding protein 3 like 1, ARID1B: AT-rich interaction domain 1B

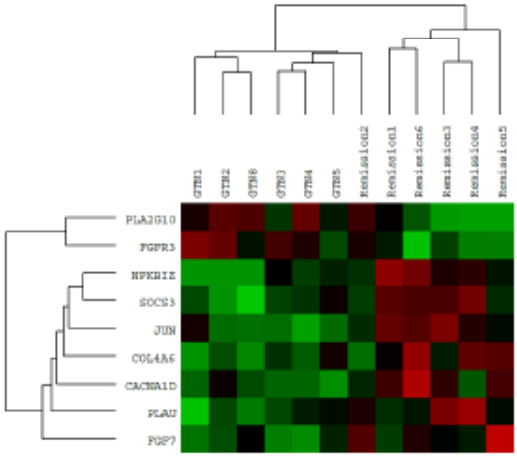

Fig. 1. Heatmap basic analysis showing the levels of mRNAs in gestational trophoblastic neoplasia moles group and the remission moles group.

Discussion

The molecular basis in GTD is not clearly understood. Evaluation of the molecular pathway driving the malignant risk in CHM is challenging. In the current study, we aimed to evaluate mRNA as a new biomarker for the prediction of postmolar GTN.

This study clearly demonstrated the differences in the profile of mRNA expression between GTN moles and remission moles. Our preliminary analysis showed that 29 mRNAs were significantly different expressed in GTN moles compared with remission moles, of which, 9 mRNAs (*JUN, COL4A6, SOCS3, PLA2G10, NFKBIZ, FGFR3, CACNA1D, FGF7* and *PLAU*) demonstrated > 2 folds differences. Therefore, we reviewed literature on the functions of these 9 mRNAs and their associations with malignancy.

JUN (c-Jun, AP-1) is a proto-oncogene. The Jun-family of proteins are critical transcription factors that act as co-activators of the androgen receptor (AR) or form activator protein 1 with fos proto-oncogene (Fos) to regulate the transcription of androgen-regulating genes. Activated c-Jun in the stromal cells plays key roles in stromal-epithelial interactions[8]. Thakur et al identified a novel binding site for activated c-Jun in the promoter of the Snail1 gene, which triggered transforming growth factor-beta (TGFβ)-induced invasion of human prostate cancer cells[9].

Next, *COL4A6* is a member of the type IV collagen family, which is a major component of the basement membrane. The basement membrane is important for confinement of the tumor microenvironment[10]. It was shown that the expression of *COL4A6* is downregulated in basal cell carcinoma, breast cancer, and colorectal carcinoma[11, 12]. Moreover, Dehan et al reported that there is a nearly complete loss of *COL4A6* protein in prostate cancer samples, but not in benign prostate hyperplasia samples using immunohistochemistry techniques[13].

SOCS3 has been studied with HER2 and STAT3 in ovarian cancer tissues. The results suggested that the increased expression of HER2 and STAT3 could promote the formation of tumor cells, and SOCS3 might inhibit tumor differentiation[14]. The outcome correlated with finding from this study which *SOCS3* had 3.09-fold higher in remission mole compared to GTN mole.

The strength of our study was the use of Nanostring nCounter technology to analyze mRNA expression. In order to detect mRNAs in the FFPE specimens, this technology has its advantages over other methods. We can digitally count mRNAs in FFPE specimens without the need for RNA amplification.

Over 770 mRNAs can be assessed in a single reaction that is suitable for cases of GTN which no known mRNA candidate was made before this study. Also, this technology has been proven to accurately assess the expression of mRNAs in both fresh frozen tissues and FFPE specimens[15-17].

Limitations of our study were a small sample size and clinical heterogeneity of the cases in both groups. However, due to the rarity of the diseases, limited studies have been performed in this field. Therefore, we primarily aimed to do this study as preliminary research to gather information on the profiles of mRNA in the GTN moles group and the remission moles group which can provide data for further replicative studies in this field.

Conclusion

In conclusion, the analysis of mRNA pancancer gene profiles can distinguish between complete hydatidiform mole that will remission from those that will turn to postmolar GTN. We identified 29 genes that were differentially expressed between the two groups. The result from this study leads to further investigations on candidate genes and could probably explain the mechanism of malignant transformation to postmolar GTN.

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Potential conflicts of interest

The authors declare no conflicts of interest.

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