Original Article

A pilot study comparing the genetic molecular biology of gestational and non-gestational choriocarcinoma

Cordelle Lazare1*, Wenhua Zhi1*, Jun Dai1, Canhui Cao1, Rajiv Rai Sookha1, Ling Wang1, Yifan Meng1, Peipei Gao1, Ping Wu1, Juncheng Wei1,2, Junbo Hu1, Peng Wu1,2

1The Key Laboratory of Cancer Invasion and Metastasis of The Ministry of Education of China, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China; 2Department of Gynecologic Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China. *Equal contributors and co-first authors.

Received July 18, 2019; Accepted October 30, 2019; Epub November 15, 2019; Published November 30, 2019

Abstract: Non-gestational choriocarcinoma (NGC) is a rare subtype of choriocarcinoma differing in origin and phenotypic characteristics compared to gestational choriocarcinoma (GC). This study aimed to analyze the molecular biology of GC and NGC and evaluate genetic anomalies of choriocarcinoma subtypes. DNA was extracted and paired from tumor-normal tissue of one NGC and one GC (control) patient for whole-exome sequencing. To further understand the role of DNAJB9, a p53 regulator mutated in the NGC tumor, on p53 upregulation in choriocarcinoma, CRISPR/Cas9 was used to induce DNAJB9 site-specific mutations in choriocarcinoma cells JEG-3. We hypothesized that DNAJB9 dysfunction would result in p53 overexpression. Sequencing revealed the GC tumor contained >7 times more somatic mutations than the NGC tumor. Missense (98.86% vs. 94.97%), stop-gain (0.57% vs. 0.93%), and frameshift mutations (0.57% vs. 4.10%) were observed in the GC and NGC samples, respectively (χ² = 24.63, P < 0.00001). The transition substitution rate was 67.54% and 55.71% in the GC and NGC samples, while the transversion substitution rate was 32.46% and 44.29% in the GC and NGC samples, respectively (χ² = 11.56, P < 0.000673). Pathway enrichment analysis revealed ECM-receptor interaction and graft-versus-host disease were most enriched in the GC and NGC tumors, respectively. In vitro investigations showed that DNAJB9 mRNA and protein levels were downregulated in Cas9-DNAJB9-sgRNA transfected cells compared to the control (P < 0.001), while p53 protein levels were upregulated. Our findings display the genetic distinctness of choriocarcinoma subtypes, especially NGC, and further highlight the relationship between p53 and DNAJB9 in choriocarcinoma cells, laying the foundation for further investigations.

Keywords: Choriocarcinoma, DNAJB9, p53, whole exome sequencing, CRISPR/Cas9

Introduction

Choriocarcinoma is a highly aggressive, malignant trophoblastic neoplasm, which is vastly curable, and typically observed in women of childbearing age [1]. This tumor displays biphasic proliferation of the two cell types making up its composition, cytotrophoblasts, and syncytiotrophoblasts, with the latter being differentiated, non-dividing, and the hormone-secreting element of the tumor [2-4]. Choriocarcinoma is subcategorized into gestational choriocarcinoma (GC) and non-gestational choriocarcinoma (NGC). GC accounts for the majority of choriocarcinoma cases, usually occurring subsequent to a molar pregnancy or infrequently follow a non-molar pregnancy, and is genetically characterized based on the presence or lack of a paternal chromosomal complement [5, 6]. The incidence rate of GC varies regionally, occurring in 1 in 20,000 to 40,000 pregnancies in western countries and 1 in 500 to 3,000 pregnancies in south-east Asia [7]. Unlike GC, NGC is not linked to any form of pregnancy and is the rarer subtype. This subtype originates from trophoblastic differentiation of germ cell tumors in the gonads or as a somatic component of another histologically different tumor such as teratoma, yolk sac tumor, embryonic carcinoma, and endometrioid adenocarcinoma; hence NGC only contains the patient’s genetic material [8-10]. Even less commonly observed is intrauterine NGC [11]. The occurrence of either choriocarcinoma subtype in postmenopausal...
Genetic similarities however with vast differences

women is extremely rare, and when encountered sparks an immediate interest in the medical community [12].

Although the phenotypic differences between these choriocarcinoma subtypes are well defined, much less is known regarding their genotypic variances [1, 6, 9]. Several studies have employed the use of microsatellite genotyping and polymorphism for differentiation between GC and NGC, GC origin determination, and pinpoint identification of the causative pregnancy in GC [10, 13-16]. Nevertheless, there has been no prior use of next-generation sequencing (NGS), more specifically whole-exome sequencing (WES), to evaluate the molecular biology of either GC or NGC.

In this study, we aimed to analyze the genetic variation profiles of GC and NGC, explicitly highlighting the shared genetic events and signaling pathway associated somatically mutated genes, and further analyze the effect of standout mutations via in vitro investigation.

Materials and methods

**Patient selection and tissue sample collection**

Two patients were identified for this study: (1) a 23-year-old female diagnosed with GC and treated with a laparoscopic hysterectomy after one cycle of neoadjuvant chemotherapy (EMA-CO); (2) a 50-year-old female (25 years following antecedent pregnancy and two years postmenopausal) diagnosed with NGC involving the left round and broad ligaments, and the left fallopian tube, surgically treated with three cycles of neoadjuvant chemotherapy (EMA-CO) followed by total transabdominal hysterectomy, bilateral adnexectomy, and cytoreduction (Table 1). Clinical staging and prognostic score were defined according to the International Federation of Gynecology and Obstetrics (FIGO) system and the prognostic scoring system of the WHO, respectively.

Archival formalin-fixed and paraffin-embedded (FFPE) tumor and adjacent non-tumor tissue samples of both patients were collected from the Department of Pathology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Histopathological diagnosis was based on the World Health Organization (WHO) classifications and executed by expert pathologists. This study was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (TJ-IRB20180612). Informed and written consent was obtained from all subjects prior to the study.

**DNA extraction and exome sequencing**

Genomic DNA (gDNA) was extracted from 30 mg of the GC and NGC tumor and adjacent non-tumor tissue samples, using the QIAmp DNA FFPE Tissue Kit (Qiagen, Germany Cat# 56404) and the RNase A Kit (Qiagen, Germany Cat# 19101) according to the manufacturer’s instructions. The DNA quality was evaluated using Qubit 3 Fluorometer (Invitrogen, USA) and agarose gel electrophoresis. The sequencing library was prepared using Aligent SureSelect All Human Exome Library (60 Mb) V6 (Agilent, USA). gDNA from each specimen was randomly sonicated to 140-200 bp and purified using magnetic beads. Fragments were then hybrid-
Genetic similarities however with vast differences

ized to the capture library. Dynal beads were subsequently used to capture the hybridized DNA fragments. The captured libraries were amplified using polymerase chain reaction (PCR) and then purified. The quality and quantity of the final library were analyzed by Qubit 3 Fluorometer and Agilent 2100 Bioanalyzer. Finally, sequencing was performed on the HiSeq X Ten (Illumina, USA).

**Data analysis**

The short reads were initially aligned using the Burrows Wheeler Aligner (BWA). Freebayes was used to recalibrate and filter alignments, and to highlight somatic mutations by comparing tumor against adjacent non-tumor tissue.

Somatic mutations were annotated by Annovar [17]. SIFT, PolyPhen2 HDIV, PolyPhen2 HVAR, and the Cosmic, Clinvar, and 1,000 Genome databases were used to predict the functional impacts of missense mutations. Variants were deemed deleterious mutations if at least two algorithms/databases scored them as deleterious. Any missense mutations which were recorded as unavailable by the algorithms/databases mentioned above were excluded from the analysis. Frameshift variants were categorized as pathogenic. All classifications used followed the standards and guidelines of the American College of Medical Genetics [18]. Circos was implemented for the visualization of overall genomic events in both tumor samples, while Maftools, an open-source R package, was implemented in lollipop plot and signature analysis visualization as previously described by Mayakonda et al. [19, 20]. All somatically mutated genes observed in this study were loaded into the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for pathway analysis [21-24]. Only pathways with $P$ values $\leq 0.05$ were included. Cytoscape was used for the visualization of these pathways [25].

**Cell line and cell culture**

The choriocarcinoma cell line, JEG-3, was used in this study (ATCC Cat# HTB-36). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) complemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 units/mL of penicillin, and were cultivated at 37°C in a humidified atmosphere containing 5% CO$_2$.

**Immunohistochemistry**

FFPE tumor tissue sections were subjected to deparaffinization and dehydration. Following H$_2$O$_2$ treatments and non-specific antigen blocking, slides were incubated with the following primary antibodies: DNAJB9 (1:50, GeneTex, USA Cat# GTX26053) and P53 (1:400, Proteintech, China Cat# 21891-1-AP) at 4°C. Subsequent to overnight incubation, the slides were incubated with secondary antibody, followed by colorimetric detection using DAB staining kit (Servicebio, China Cat# G1211). Negative controls were prepared by replacing the primary antibodies with phosphate-buffered saline (PBS). The intensity of immunohistochemistry staining was determined based on five random microscopic fields. Numeric scores were assigned on the percentage of cells stained: 0 (< 5%), 1 (5%-25%), 2 (26%-50%), and 3 (51-75%), and 4 (76-100%). Numeric values were also assigned to express immunohistochemistry staining intensity: 0 (colorless), 1 (light yellow), 2 (brownish yellow), and 3 (brown). Expression was determined by the multiplication of both scores per slide with a final score of 0 representing negative expression (-), while scores 1-4, 5-8, and 9-12 represented weak positivity (+), positive (++) and strong positivity (+++), respectively.

**Transfection and induction of DNAJB9 dysfunction via CRISPR/Cas9**

To further explore the function of DNAJB9 in choriocarcinoma cell lines DNAJB9 site-specific genetic alterations were carried out as follows: JEG-3 cells were transfected with lentivirus expressing either one of two single guide RNA (sgRNA), DNAJB9-sgRNA1-GFP or DNAJB9-sgRNA2-GFP, each targeting a single nucleotide of the second exon of the DNAJB9 gene, and co-expressing nCas9. The sgRNA sequences are as follows: sgRNA1: 5’-TATCTTAGGTGTGCCAAAAAT-3’; sgRNA2: 5’-TGTGAAAGGCCTTCTTATTG-ATT-3’.

JEG-3 cells transfected with empty lentivirus were used as negative control. Lentiviruses were obtained from ViGene Biosciences (Shandong, China).

**T7 endonuclease 1 enzyme assay**

In order to perform PCR proliferation, cells were collected following transfection, and total DNA was extracted from cells using the E.Z.N.A
Tissue DNA Kit (Omega Bio-tek, USA Cat# D3396-01) according to the vendor’s protocol. PCR products were then purified using E.Z.N.A Cycle-Pure Kit (Omega Bio-tek, USA Cat# D6492-02) based on instructions provided by the manufacturer. The DNAJB9 primer sequence is as follows: Forward: 5’-TCTCCTCTGTGTATGGCCAGA-3’; Reverse: 5’-TGCTCAGCGTGCAATTTG-3’.

Targeting efficiencies were measured using the T7 Endonuclease I (T7E1) Kit (New England BioLabs, USA Cat# M0302S) following the manufacturer’s instructions. Detection was performed using agarose gel electrophoresis.

Reverse transcription quantitative real-time PCR for the detection of DNAJB9

Total RNA was extracted from the cells using TRizol Reagent (Invitrogen, USA Cat# 15596026) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Takara, Japan). Reverse transcription quantitative real-time PCR (RT-qPCR) was performed using a Bio-Rad CFX96 system with SYBR Green. The DNAJB9 primer sequence is as follows: Forward: 5’-ATCTTAGGTGTGCAATTTG-3’; Reverse: 5’-GAACAAAAAGCAGGACTTT-3’.

The reactions were amplified as follows: 95°C for 3 mins and 40 cycles of 95°C for 10 s, 60°C for 30 s, and 95°C for 15 s.

Western blot analysis

Total cell lysates were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer (Be-yotime, China) supplemented with a protease inhibitor cocktail (Roche, Germany). Protein concentrations were determined using Coomassie blue staining. Total lysates (40 μg per sample) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
Genetic similarities however with vast differences

followed by immunoblotting with primary antibodies against the following proteins: DNAJB9 (1:500, GeneTex, USA Cat# GTX26053), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:4,000, Proteintech Group, China Cat# 60-004-1-Ig), and TP53 (1:500, ABclonal, USA Cat# A5761). After the membranes were washed, they were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (antGENE, China), and the proteins were detected using an enhanced chemiluminescence system (Thermo Fisher Scientific, USA).

Statistics

The data are presented as the mean value ± standard error of mean (SEM) acquired from at least three independent experiments. Prism 6.0 GraphPad Software was used to perform statistical analyses. Single comparisons between two groups were done by Student’s t-test. Comparison of the ratio of various mutations between GC and NGC was done by Chi-square test. P values of ≤ 0.05 were considered statistically significant.

Results

Summary of somatic mutations

Summaries of variants identified in both tumor samples are shown in Figure 1. Overall, 1574 and 219 somatic nonsynonymous variants in exons were observed in the GC and NGC tumor samples, respectively. Among the somatic non-
Genetic similarities however with vast differences

synonymous variants in the GC tumor, 1556 (98.86%) missense mutations, 9 (0.57%) stop-gain mutations, and 9 (0.57%) frameshift mutations were observed, while 208 (94.97%) missense mutations, 2 (0.93%) stop-gain mutations, and 9 (4.10%) frameshift mutations were observed in the NGC tumor ($x^2 = 24.63, P < 0.00001$) (Figure 2A and 2B). The overall rate of transition substitution was 67.54% (1057/1565) and 55.71% (117/210) in the GC and NGC samples, respectively, while the rate of transversion substitution was 32.46% (508/1565) and 44.29% (93/210) in the GC and NGC samples, respectively ($x^2 = 11.56, P < 0.000673$). Although the transition and transversion substitution counts displayed astonishing differences between the GC and NGC tumors, smaller discrepancies were observed in the actual rates of these two substitution types (Figure 2C and 2D). For instance, 194 and 31 A > G transition substitutions were observed in the GC and NGC samples, respectively. However, this substitution accounted for 12.40% (194/1565) and 14.76% (31/208) in the GC and NGC samples, respectively. 71 and 11 C > A transversion substitutions were highlighted in the GC and NGC samples, respectively. As per the above trend, this substitution accounted for 4.54% (71/1565) and 5.24% (11/210) in the GC and NGC samples, respectively. A similar trend was observed with further analysis using the algorithms/databases as mentioned earlier for variant prediction (Figure 2E and 2F). Though the number of variants predicted to be either benign/neutral or deleterious in the GC sample far exceeded those in the NGC sample, the percentage of either prediction in both samples was staggeringly similar. Benign/neutral variants accounted for 75.98% (1196/1574) and 72.15% (158/219) in the GC and NGC samples, respectively, in contrast to 24.02% (378/1574) and 27.85% (61/219) of variants being deleterious in the GC and NGC samples, respectively ($x^2 = 1.53, P = 0.22$). All frameshift mutations were classified as pathogenic.

Multiple gene family mutations were observed in both tumors. Of these, 75 zinc finger (ZNF), 35 solute carrier (SLC), and seven mucin (MUC) genes were mutated in the GC tumor. Of the multiple gene family mutations in the NGC tumor, most noteworthy were 18 MUC and 4 ZNF genes. Twenty-seven somatic mutations (26 missense mutations, one frameshift mutation) were shared between these two tumors (Table 2). Among the somatic nonsynonymous variants of the NGC tumor, two were missense mutations of the second exon of the DNAJB9 gene (c.T137A:p.F46Y, c.A140G:p.H47R) (Figure 3A).

Mutational signatures and KEGG pathway analysis

Mutational analysis revealed two signatures between GC and NGC, which were characterized by C > T mutations (Figure 3B). Signature 1 related to the spontaneous deamination of 5-methylcytosine, while signature 5 was of unknown etiology. However, due to limited sample size, further characterization in additional cases is required.

All somatic mutations observed in the GC (1574) and NGC (219) were subjected to enrichment analysis with the KEGG pathways. We further found that genes in the GC sample were enriched in the following: olfactory transduction, ECM-receptor interaction, protein digestion and absorption, and amoebiasis, while genes in the NGC sample were enriched in the following: olfactory transduction, phagosome, graft-versus-host disease, antigen processing and presentation, and allograft rejection (Figure 4A-D; Table 3). Additionally, the most significant pathway was olfactory transduction in both GC and NGC samples.

DNAJB9 and p53 expression in GC and NGC samples

DNAJB9, observed to be mutated in the NGC tumor, is a known negative feedback regulator of the tumor suppressor gene p53, a tumor suppressor gene which is interestingly known to display upregulated expression in choriocarcinoma [1, 26]. Hence, in light of the relationship between DNAJB9 and p53, we investigated the effects of DNAJB9 mutations on p53 expression in choriocarcinoma. Figure 5A represents the immunohistochemistry staining for DNAJB9 and p53 in the GC and NGC tumor samples. DNAJB9 was observed to be mainly expressed in the cell membrane and cytoplasm. Based on the scoring method used, DNAJB9 expression levels in the GC sample displayed strong positivity (+++), while weak positivity (+) was observed in the NGC sample.
Table 2. Shared mutations between gestational choriocarcinoma and non-gestational choriocarcinoma samples

<table>
<thead>
<tr>
<th>Chr</th>
<th>Position</th>
<th>Reference Allele</th>
<th>Tumor Allele</th>
<th>Zygosity</th>
<th>dbSNP ID</th>
<th>Region</th>
<th>Type</th>
<th>Gene</th>
<th>Transcript Name</th>
<th>Exon</th>
<th>CDS Position</th>
<th>Protein Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>73575485</td>
<td>A</td>
<td>G</td>
<td>Hetero</td>
<td>rs7494</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>ACOT2</td>
<td>NM_006821</td>
<td>3</td>
<td>c.A1424G</td>
<td>p.H475R</td>
</tr>
<tr>
<td>2</td>
<td>240682792</td>
<td>T</td>
<td>C</td>
<td>Hetero</td>
<td>rs4081908</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>AQP12B</td>
<td>NM_001102467</td>
<td>1</td>
<td>c.A46</td>
<td>p.T16A</td>
</tr>
<tr>
<td>12</td>
<td>107319734</td>
<td>G</td>
<td>C</td>
<td>Hetero</td>
<td>rs961498</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>BTBD11</td>
<td>NM_001018072</td>
<td>1</td>
<td>c.G794C</td>
<td>p.G265A</td>
</tr>
<tr>
<td>1</td>
<td>247111597</td>
<td>A</td>
<td>G</td>
<td>Hetero</td>
<td>rs73135916</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>C1orf229</td>
<td>NM_207401</td>
<td>1</td>
<td>c.T628C</td>
<td>p.S210P</td>
</tr>
<tr>
<td>5</td>
<td>13931231</td>
<td>C</td>
<td>T</td>
<td>Hetero</td>
<td>rs1530049</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>DNAH5</td>
<td>NM_001369</td>
<td>2</td>
<td>c.G71A</td>
<td>p.G24E</td>
</tr>
<tr>
<td>3</td>
<td>75665689</td>
<td>A</td>
<td>G</td>
<td>Hetero</td>
<td>rs200756071</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>FRG2C</td>
<td>NM_001124759</td>
<td>4</td>
<td>c.A497G</td>
<td>p.E166G</td>
</tr>
<tr>
<td>15</td>
<td>34386710</td>
<td>C</td>
<td>G</td>
<td>Hetero</td>
<td>rs147828722</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>GOLGA8A</td>
<td>NM_181077</td>
<td>3</td>
<td>c.G200C</td>
<td>p.R67P</td>
</tr>
<tr>
<td>6</td>
<td>31356423</td>
<td>G</td>
<td>C</td>
<td>Hetero</td>
<td>rs1140412</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>HLA-B</td>
<td>NM_005514</td>
<td>3</td>
<td>c.C363G</td>
<td>c.C363G</td>
</tr>
<tr>
<td>17</td>
<td>20467454</td>
<td>G</td>
<td>C</td>
<td>Hetero</td>
<td>rs4985834</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>LGALS9B</td>
<td>NM_001042685</td>
<td>1</td>
<td>c.C17G</td>
<td>p.S6C</td>
</tr>
<tr>
<td>14</td>
<td>39246997</td>
<td>A</td>
<td>G</td>
<td>Hetero</td>
<td>rs11845046</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>MIA2</td>
<td>NM_054024</td>
<td>4</td>
<td>c.A423G</td>
<td>p.I141M</td>
</tr>
<tr>
<td>9</td>
<td>94325541</td>
<td>G</td>
<td>C</td>
<td>Hetero</td>
<td>rs20099818</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>NUTM2F</td>
<td>NM_017561</td>
<td>2</td>
<td>c.G410G</td>
<td>p.S137C</td>
</tr>
<tr>
<td>2</td>
<td>130074612</td>
<td>G</td>
<td>A</td>
<td>Hetero</td>
<td>rs62165870</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>POTEF</td>
<td>NM_00109977</td>
<td>17</td>
<td>c.C2860T</td>
<td>p.R954W</td>
</tr>
<tr>
<td>2</td>
<td>240042210</td>
<td>A</td>
<td>G</td>
<td>Hetero</td>
<td>rs200217866</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>PRR21</td>
<td>NM_001080835</td>
<td>1</td>
<td>c.T773C</td>
<td>p.M258T</td>
</tr>
<tr>
<td>2</td>
<td>240042322</td>
<td>A</td>
<td>G</td>
<td>Hetero</td>
<td>rs200217866</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>PRR21</td>
<td>NM_001080835</td>
<td>1</td>
<td>c.T661C</td>
<td>p.C221R</td>
</tr>
<tr>
<td>9</td>
<td>2828765</td>
<td>C</td>
<td>G</td>
<td>Hetero</td>
<td>rs2173904</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>PUM3</td>
<td>NM_014878</td>
<td>9</td>
<td>c.G866C</td>
<td>p.R289P</td>
</tr>
<tr>
<td>12</td>
<td>100338529</td>
<td>C</td>
<td>G</td>
<td>Hetero</td>
<td>rs56391464</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>SCYL2</td>
<td>NM_017988</td>
<td>18</td>
<td>c.C2147G</td>
<td>p.T716S</td>
</tr>
<tr>
<td>19</td>
<td>4606668</td>
<td>C</td>
<td>G</td>
<td>Hetero</td>
<td>rs18971417</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>SHC2</td>
<td>NM_012435</td>
<td>1</td>
<td>c.G329C</td>
<td>p.G110A</td>
</tr>
<tr>
<td>6</td>
<td>167180694</td>
<td>A</td>
<td>C</td>
<td>Hetero</td>
<td>rs1537172</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>TCP10L2</td>
<td>NM_00114512</td>
<td>7</td>
<td>c.A831C</td>
<td>p.E277D</td>
</tr>
<tr>
<td>13</td>
<td>23593366</td>
<td>T</td>
<td>A</td>
<td>Hetero</td>
<td>rs9550987</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>TNFRSF19</td>
<td>NM_001204458</td>
<td>3</td>
<td>c.T91A</td>
<td>p.S31T</td>
</tr>
<tr>
<td>2</td>
<td>178658787</td>
<td>T</td>
<td>A</td>
<td>Hetero</td>
<td>rs200021871</td>
<td>Exonic</td>
<td>Nonsyn</td>
<td>TTN</td>
<td>NM_001267550</td>
<td>183</td>
<td>c.A37461T</td>
<td>p.E12487D</td>
</tr>
<tr>
<td>7</td>
<td>151086813</td>
<td>G</td>
<td>Hetero</td>
<td>rs539642617</td>
<td>Exonic</td>
<td>Fshift</td>
<td>AGAP3</td>
<td>NM_001042535</td>
<td>1</td>
<td>c.73dupG</td>
<td>p.G24fs</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Chr: chromosome; Fshift: frameshift; Hetero: heterozygous; Nonsyn: nonsynonymous.
In contrast, p53 was observed to be mainly expressed in the nucleus and partly in the cytoplasm. The GC sample displayed weak positivity (+) for p53, while the NGC sample displayed strong positive (+++) p53 expression.

**DNAJB9 dysfunction upregulates p53 expression in choriocarcinoma**

To explore the function of the DNAJB9 gene in choriocarcinoma, we designed two sgRNAs (DNAJB9-sgRNA1-GFP and DNAJB9-sgRNA2-GFP) to achieve site-specific mutations in DNAJB9 in JEG-3 cells. Successful transfection of designed sgRNAs was confirmed with GFP expression observed via a confocal microscope (Figure 5B). The T7E1 Enzyme Assay confirmed site-specific mutations induced by the CRISPR/Cas9 gene-editing system (Figure 5C). RT-qPCR performed to analyze DNAJB9 mRNA expression levels showed that downregulation followed site-specific mutations induced by CRISPR/Cas9 gene-editing system (Figure 5D). It was observed that DNAJB9 protein expression levels were also downregulated, while p53 protein expression levels were upregulated following the induction of these site-specific DNAJB9 mutations (Figure 5E). These findings taken together prove that site-specific DNAJB9 mutations induced by the CRISPR/Cas9 gene-editing system induced DNAJB9 gene dysfunctions, thus down-regulating DNAJB9 expression which subsequently led to the upregulated expression of p53 in JEG-3 cells.

**Discussion**

In the current study, we report a pilot analysis of genomic alterations in GC and NGC using WES and put forth the first study of this nature. In our analysis, multiple mutations in the ZNF, SLC, and MUC gene families were observed in both tumor samples, with the GC tumor containing a minimum of twice the number of mutations among these gene families compared to the NGC tumor. These gene families play roles in transcriptional regulation and the coding of transporters and have been linked to several diseases, including cancer [27-29]. None of the above mutations have been previously associated with choriocarcinoma and may warrant further study.

A keen observation of this study was the overwhelming disparity in the overall number of somatic mutations between the GC and NGC samples when analyzed based on the aforementioned categories. However, both the GC and NGC samples displayed similar total percentages in every category. GC may be androgenic or biparental in its genetic composition [6]. Nevertheless, this case of GC was diagnosed less than one year following an antecedent pregnancy, a key factor for proceeding with the notion that this case of GC was biparental in its genetic composition. Such a genetic makeup would explain the large number of somatic mutations far exceeding that of NGC. In contrast, NGC is unrelated to any form of gestation and is, therefore, genetically linked to the patient [6]. This may explain fewer somatic mutations observed in our NGC tumor. Notwithstanding the genetic composition of NGC, it could contain karyotypic aberrations [9]. Nonetheless, both samples displayed similar ratios of somatic mutations across all categories analyzed. Hence, we believe that alth-
Genetic similarities however with vast differences though the origin of choriocarcinoma plays a fundamental role in its genetic composition, both subtypes may be similar in regards to the ratio of different genetic events, possibly meaning that the number and type of genetic events may not have a direct influence on this disease. Specific genetic anomalies occurring in key genes may be a more significant factor in determining the phenotypic characteristics of GC and NGC.

Figure 4. A. Bar chart representation of the enrichment mutated genes observed in the GC tumor in KEGG pathways. B. Bar chart representation of the enrichment mutated genes observed in the NGC tumor in KEGG pathways. C. Cytoscape representation of mutated genes found in the GC sample in KEGG pathways. D. Cytoscape representation of mutated genes observed in the NGC sample in KEGG pathways.
Genetic similarities however with vast differences

Table 3. KEGG pathway analysis of somatic mutations in GC and NGC samples

<table>
<thead>
<tr>
<th>Pathway ID</th>
<th>GOTerm</th>
<th>GC Pathway ID</th>
<th>P-value</th>
<th>Associated Genes (%)</th>
<th>N. of Genes</th>
<th>Associated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04740</td>
<td>Olfactory transduction</td>
<td>5.0E-4</td>
<td>9.27</td>
<td>37</td>
<td>ADCY3, CNGB1, OR1A2, OR10G4, OR13D1, OR13F1, OR2C1, OR2C3, OR2F1, OR2J2, OR2T29, OR2T34, OR2T8, OR3Z2, OR4C46, OR4D2, OR4D11, OR4F21, OR4K1, OR4K5, OR5B12, OR5H6, OR5M1, OR5V1, OR5Z41, OR6B1, OR6C, OR6F1, OR6K3, OR6X1, OR7D4, OR7E24, OR8B2, OR8S1</td>
<td></td>
</tr>
<tr>
<td>hsa04512</td>
<td>ECM-receptor interaction</td>
<td>4.1E-4</td>
<td>16.10</td>
<td>14</td>
<td>COL3A1, COL4A4, COL6A5, COL6A6, COL24A1, COL27A1, ITGA11, LAMA2, LAMA3, LAMC4, LAMG4, LAMB4, LAMC3, SDC4, TNN</td>
<td></td>
</tr>
<tr>
<td>hsa04974</td>
<td>Protein digestion and absorption</td>
<td>4.8E-3</td>
<td>13.64</td>
<td>12</td>
<td>COL3A1, COL4A4, COL6A5, COL6A6, COL17A1, COL18A1, COL24A1, COL27A1, CRCP, PRSS3, SLC3A1, SLC3A4</td>
<td></td>
</tr>
<tr>
<td>hsa05146</td>
<td>Amoebiasis</td>
<td>4.3E-2</td>
<td>12.64</td>
<td>11</td>
<td>COL3A1, COL4A4, COL17A1, COL18A1, C8A, LAMA2, LAMA3, LAMC4, LAMG4, LAMC3, SERPINB10</td>
<td></td>
</tr>
<tr>
<td>NGC</td>
<td>hsa04740</td>
<td>Olfactory transduction</td>
<td>8.6E-5</td>
<td>3.01</td>
<td>12</td>
<td>OR1Q1, OR10H1, OR2M2, OR2T34, OR2T8, OR4N2, OR5H2, OR5K3, OR5L2, OR6B3, OR6C6, OR8B3</td>
</tr>
<tr>
<td>hsa04145</td>
<td>Phagosome</td>
<td>6.3E-4</td>
<td>4.67</td>
<td>7</td>
<td>DYNC2H1, HLA-A, HLA-B, HLA-DQB1, HLA-DRB1, SFTPAP1, SFTPAP2</td>
<td></td>
</tr>
<tr>
<td>hsa05332</td>
<td>Graft-versus-host disease</td>
<td>1.6E-3</td>
<td>12.12</td>
<td>4</td>
<td>HLA-A, HLA-B, HLA-DQB1, HLA-DRB1</td>
<td></td>
</tr>
<tr>
<td>hsa04612</td>
<td>Antigen processing and presentation</td>
<td>2.0E-3</td>
<td>6.58</td>
<td>5</td>
<td>HSPA2, HLA-A, HLA-B, HLA-DQB1, HLA-DRB1</td>
<td></td>
</tr>
<tr>
<td>hsa05330</td>
<td>Allograft rejection</td>
<td>2.2E-3</td>
<td>10.81</td>
<td>4</td>
<td>HLA-A, HLA-B, HLA-DQB1, HLA-DRB1</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: GOTerm: gene ontology term.
Genetic similarities however with vast differences

Our results also showed that the GC sample contained mutated genes which were most enriched in ECM-receptor interaction. Previous reports have highlighted that complex ECM interactions play pivotal roles in the regulation of homeostasis, inflammation, morphogenesis, proliferation, differentiation, and migration [30, 31]. Any dysregulation of these interactions induces a host of pathological conditions, including invasive cancer [32]. Here, the dysfunction of ECM interactions further stimulates cancer progression via cancer cell polarity, signaling, and increased motility [33]. Moreover, both the GC and NGC samples contained mutated genes which were highlighted to be enriched in olfactory transduction. Olfactory receptors (ORs) are members of G-protein coupled receptors, the largest gene family in the human genome [34, 35]. Previously, ORs were thought to only function in the olfactory epithelium and were ignored in regards to tumorigenesis. Recent studies have since refuted this notion and proven that ORs are linked to several cancers and influence many levels of carcinogenesis, including proliferation, migration, invasion, etc. [34, 36-38].

Interestingly, the NGC tumor contained mutated genes most enriched in graft-versus-host disease and highly enriched in allograft rejection. Previous reports have highlighted GC as a tissue allograft, in contrast to NGC, which originated from the patient [6]. Thus, findings of genetic mutations associated with these pathways in the NGC tumor are rather peculiar. Therefore, we propose that dysfunction of these pathways may be responsible for the phenotypic characteristics of choriocarcinoma, namely its highly metastatic nature, and that the identification of these pathways should prompt further investigation of these pathways for targeted treatment of choriocarcinoma. Additionally, the roles of graft-versus-host disease and allograft rejection pathways in choriocarcinoma, especially NGC, should be further evaluated.

**Figure 5.** (A) Immunohistochemistry staining (×200) for DNAJB9 and p53. (B) Expression of DNAJB9-sgRNA1-GFP and DNAJB9-sgRNA2-GFP in JEG-3 cell detected using an inverted fluorescence microscope (×200). (C) The detection of targeted deletion mutation of DNAJB9 in which sgRNA1 produced 300-bp and 580-bp products, and sgRNA2 produced 340-bp and 540-bp products. (D) DNAJB9 mRNA levels were assessed in Cas9 + DNAJB9-sgRNA1, Cas9 + DNAJB9-sgRNA2, and empty lentivirus (control) JEG-3 cells using RT-qPCR. (E) Western blotting analysis confirmed DNAJB9 downregulation and p53 overexpression in JEG-3 cells transfected with Cas9 + DNAJB9-sgRNA1 and Cas9 + DNAJB9-sgRNA2 compared to control. Every bar represents the mean ± SEM of three independent experiments in (D). ***P < 0.001 by Student’s t-test.
A well-known correlation has been drawn between the mutations of the tumor suppressor gene p53 and many cancers. Intriguingly, no p53 mutations were observed in either choriocarcinoma sample. However, several studies have highlighted p53 expression to be upregulated in choriocarcinoma [1, 39]. Many studies have explored p53 regulation and have highlighted several genes which inhibit p53 function by acting as negative feedback regulators [40-42]. Among these is DNAJB9, a gene which regulates ATPase activity of Hsp70s [43-45]. According to Lee et al., p53 induces DNAJB9 expression, which then acts as a negative feedback regulator of p53 via the Ras/Raf/ERK pathway and nuclear interaction [26]. Astonishingly, we found two mutations of the DNAJB9 gene in the NGC sample, the only of these p53 regulator genes mutated in either choriocarcinoma sample. Therefore, in order to further investigate the relationship between DNAJB9 mutation and p53 overexpression in choriocarcinoma, we induced site-specific DNAJB9 mutations and demonstrated the effect of these mutations on p53 expression in JEG-3 cells. Compared with the control group, DNAJB9 mRNA and protein expression levels in JEG-3 cells were downregulated in both Cas9 + DNAJB9-sgRNA transfected groups. We further confirmed that compared to the control group, p53 protein expression levels in JEG-3 cells were upregulated in both Cas9 + DNAJB9-sgRNA transfected groups. These findings demonstrated that site-specific DNAJB9 mutations induced by CRISPR/Cas9 triggered DNAJB9 gene dysfunction, thus interrupting its negative feedback regulation of p53, finally leading to p53 overexpression, hence a probable cause of p53 upregulation in choriocarcinoma. This also further highlights the relationship between DNAJB9 and p53.

However, this study is not without limitations. First, this study contains a small sample size. We do believe that in light of this limitation and our exciting discoveries, there is a need for further investigation involving a larger sample size in which these two types of choriocarcinoma are further genetically analyzed. Moreover, we were unable to gain access to paternal DNA in order to eliminate paternal germline mutations in the GC case. We propose that further investigations include the elimination of paternal germline mutations in order to only identify and analyze somatic mutations.

In conclusion, this study represents a pilot effort which used NGS to gain a much better understanding of the molecular biology of the choriocarcinoma as both GC and NGC are molecularly distinct, and will undoubtedly provide much guidance as we embark on larger related projects. We identified several somatically mutated genes in either type of choriocarcinoma with specific mutations being presented as possible factors in the clinical characteristics of choriocarcinoma. Additionally, via the use of CRISPR/Cas9, we further underscored the relationship between DNAJB9 and p53 and presented DNAJB9 mutations as a possible causative factor in p53 upregulation in the JEG-3 choriocarcinoma cell line.

Acknowledgements

This work was supported by the National Science Foundation of China (grant numbers 81630060; 81772775); the National Key Research and Development Program of China (grant number 2016YFC0902901) and the National 973 Project of China (grant number 2015CB553903).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Peng Wu, Department of Gynecologic Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China. Tel: +86-13995573729; E-mail: pengwu8626@tjh.tjmu.edu.cn

References


Genetic similarities however with vast differences


