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<td>Author(s)</td>
<td>Fong, PY; Xue, WC; Ngan, HYS; Chiu, PM; Chan, KYK; Tsao, SW; Cheung, ANY</td>
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P-Y Fong, W-C Xue, H Y S Ngan, P-M Chiu, K Y K Chan, S W Tsao and A N Y Cheung

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Caspase activity is downregulated in choriocarcinoma: a cDNA array differential expression study

P-Y Fong, W-C Xue, H Y S Ngan, P-M Chiu, K Y K Chan, S W Tsao, A N Y Cheung

Background: Placental trophoblast can be considered to be pseudomalignant tissue and the pathogenesis of gestational trophoblastic diseases remains to be clarified.

Aims: To examine the role of caspases 8 and 10, identified by differential expression, on trophoblast tumorigenesis.

Methods: cDNA array hybridisation was used to compare gene expression profiles in choriocarcinoma cell lines (JAR, JEG, and BeWo) and normal first trimester human placentas, followed by confirmation with quantitative real time polymerase chain reaction and immunohistochemistry. Caspase 10 and its closely related family member caspase 8 were analysed.

Results: Downregulation of caspase 10 in choriocarcinoma was detected by both Atlas human cDNA expression array and Atlas human 1.2 array. Caspase 10 mRNA expression was significantly lower in hydatidiform mole (p = 0.035) and chorioarcoma (p = 0.002) compared with normal placenta. The caspase 8 and 10 proteins were expressed predominantly in the cytotrophoblast and syncytiotrophoblast, respectively, with significantly lower expression in choriocarcinomas than other trophoblastic tissues (p < 0.05). Immunoreactivity for both caspase 8 and 10 correlated with the apoptotic index previously assessed by terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (p = 0.02 and p = 0.04, respectively) and M30 (p < 0.001 and p = 0.003, respectively) approaches.

Conclusions: These results suggest that the downregulation of caspases 8 and 10 might contribute to the pathogenesis of choriocarcinoma.
Pooled RNA from three first trimester placentas (gestational age, 8–10 weeks) and three CCA cell lines were used for the cDNA array experiments. For the real time RT-PCR experiments, nine first trimester placentas (gestational age, 7–12 weeks), two second trimester placentas (gestational age, 18–20 weeks), and four term placentas (gestational age, 38–41 weeks), in addition to 13 HMs that regressed (gestational age, 8–13 weeks), 13 term placentas (gestational age, 38–41 weeks), and 11 spontaneous abortions (gestational age, 5–12 weeks), were also included. Thirty two of the HMs spontaneously regressed after suction evacuation, whereas 16 patients developed GTN and required chemotherapy. The diagnosis in most cases was confirmed by fluorescent microsatellite genotyping after microdissection and with chromosome in situ hybridisation to analyse ploidy status.

RNA extraction and cDNA array analysis
Total RNA was extracted and the mRNA was then isolated using the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Frozen sections were histologically assessed to ensure that over 90% of the tissue was trophoblast. Equal amounts of pooled mRNA from three normal first trimester placentas (gestational age, 8–10 weeks) and three cell lines were used for probe synthesis. The differential gene expression experiments used the commercially available Atlas™ human cDNA expression array and the Atlas™ human 1.2 array (Clontech, Palo Alto, California, USA), which analysed 588 and 1176 cDNA tags, respectively, related to a wide range of biological pathways. The probes were labelled with [³²P]dATP, hybridised on to the membranes, washed, and exposed to x ray films. The intensities of the dotted genes were measured with AtlasImage™ (Clontech).

Real time RT-PCR
Primers and TaqMan probes were designed to span three exons so that they were specific to the target transcripts and to eliminate amplification from genomic DNA. Caspase 8: (exon 6) forward primer, 5'CCG CAA AGG AAG CAA GAA C-3'; (exon 8) reverse primer, 5'AAT TCT GAT CTG CTC ACT TCT TCT G-3'; and TaqMan probe, 5'FAM-TGC CTT GAT GTT ATT CCA GAG ACT CCA GGA-TAMRA-3' (accession number NM_001228). Caspase 10: (exon 3) forward primer, 5'CAG AAG GCA TTG ACT CAG AGA ACT-3'; (exon 5) reverse primer, 5'GAT ACG ACT CGG CTT CCT TGT-3'; and TaqMan probe, 5'FAM-TGG CCT GAT GTT ATT CCA GAG ACT CCA GGA-TAMRA-3' (accession number NM_032974). The expression of caspases 8 and 10 was normalised against the expression of the housekeeping gene, TBP (TATA box binding protein). The quantitative real time reaction was carried out and analysed using the ABI7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA).

Immunohistochemical staining of caspases 8 and 10
Paraffin wax embedded sections were incubated with primary goat polyclonal anticaspase 8 (p20; C-20; Santa Cruz Biotech, Santa Cruz, California, USA) and rabbit polyclonal anticaspase 10 (Cell Signaling Technology, Beverly, California, USA). The sections were then developed with biotinylated secondary antibody (Amersham, Piscataway, New Jersey, USA), followed by streptavidin–biotin peroxidase complex (Dako, Glostrup, Denmark). Freshly prepared 3, 3-diaminobenzidine was used for colour development and the sections were then counterstained with Mayer's haematoxylin.
Immunostaining for caspases 8 and 10 was scored with respect to relative intensity on an arbitrary scale: 1, weak; 2, moderate; and 3, intense. The percentage of positive cells was also estimated. The values of these two parameters were multiplied to produce an immunoreactive score, which ranged from 0 to 300 for each case.

**Statistical analysis**
Correlations between numerical data were performed using Pearson’s correlation test. Numerical data that were not normally distributed were analysed by the Mann-Whitney test and analysis of variance. A p value less than 0.05 was taken as significant.

**RESULTS**

**Downregulation of caspase 10 mRNA expression in CCA cell lines by cDNA array analysis**
Genes with differential expression were defined as upregulated or downregulated if they displayed a twofold difference in expression. We found a twofold downregulation of caspase 10 using both the Atlas human cDNA expression array and the Atlas human 1.2 array (data not shown).

**Reduced expression of caspases 8 and 10 in HM and CCA**
Quantitative real time RT-PCR confirmed the reduced expression of caspase 10 in HM (p = 0.035) and CCA (p = 0.002) when compared with normal placenta (data not shown).

Immunohistochemical studies show that immunoreactivity for caspases 8 (fig 1) and 10 (fig 2) was found predominantly in the cytoplasm of the cytotrophoblast (CT) and syncytiotrophoblast (ST), respectively, in all the trophoblasts studied. No expression of caspase 8 was found in STs, whereas only weak expression of caspase 10 was found in CTs. Immunostaining for caspases 8 and 10 was weak in the intermediate trophoblast infiltrating the decidua.

The amount of caspase 8 and caspase 10 protein was significantly lower (p < 0.05; tables 1–3) in CCA than in normal placenta, spontaneous abortion, partial HM, and complete HM. However, there were no significant difference among the last four categories except for the marginal difference in caspase 8 expression between normal placenta and spontaneous abortion.

The expression of caspases 8 and 10 was not significantly different between the HMs that subsequently regressed and those that developed persistent GTN, with or without metastasis (p = 0.13 and p = 0.5, respectively). Therefore, there was no correlation between the expression of caspases 8 and 10 and the clinical behaviour of HM.

**Correlation with apoptotic markers**
The results were also correlated with our previous findings on apoptotic activity in matched samples. Caspase 8 and 10

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**Table 1** Caspase 8 index of normal placenta, spontaneous abortion, hydatidiform mole, and choriocarcinoma

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<tr>
<th></th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
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<tr>
<td>Normal placenta (n = 27)</td>
<td>40–225</td>
<td>139</td>
<td>65.9</td>
</tr>
<tr>
<td>Spontaneous abortion (n = 11)</td>
<td>5–180</td>
<td>71</td>
<td>50.5</td>
</tr>
<tr>
<td>Partial mole (n = 14)</td>
<td>40–225</td>
<td>115</td>
<td>63.8</td>
</tr>
<tr>
<td>Complete mole (n = 34)</td>
<td>20–240</td>
<td>115</td>
<td>55.4</td>
</tr>
<tr>
<td>Choriocarcinoma (n = 7)</td>
<td>15–60</td>
<td>30.7</td>
<td>15.4</td>
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**Table 2** Caspase 10 index of normal placenta, spontaneous abortion, hydatidiform mole, and choriocarcinoma

<table>
<thead>
<tr>
<th></th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
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<tbody>
<tr>
<td>Normal placenta (n = 27)</td>
<td>60–150</td>
<td>120</td>
<td>26.2</td>
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<tr>
<td>Spontaneous abortion (n = 11)</td>
<td>40–240</td>
<td>110</td>
<td>53.7</td>
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<td>Partial mole (n = 14)</td>
<td>60–255</td>
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<td>64</td>
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<tr>
<td>Complete mole (n = 34)</td>
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<tr>
<td>Choriocarcinoma (n = 7)</td>
<td>10–55</td>
<td>34.2</td>
<td>17.4</td>
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The expression of these caspases map to the same region of chromosome 2q33–34, so that they might arise from the duplication of one ancestral gene. Shikama detected both caspase 8 and caspase 10 in the cytoplasm of HeLa cancer cells. The expression of procaspases 8 and 10 in tumour cell lines was associated with TRAIL (tumour necrosis factor related apoptosis inducing ligand) induced apoptosis and was important in the resistance to chemotherapy. Moreover, caspase 10 expression was found to be more abundant in fetal lung, kidney, and skeletal muscle but not in the corresponding adult tissues, suggesting that caspase 10 expression was limited to the placenta cell line as control. Caspase 10, an initiator of death receptor signalling, is closely related to the sequence of caspase 8. The genes for these two molecules map to the same region of chromosome 2q33–34, so that they might arise from the duplication of one ancestral gene. Shikama detected both caspase 8 and caspase 10 in the cytoplasm of HeLa cancer cells. The expression of procaspases 8 and 10 in tumour cell lines was associated with TRAIL (tumour necrosis factor related apoptosis inducing ligand) induced apoptosis and was important in the resistance to chemotherapy. Moreover, caspase 10 expression was found to be more abundant in fetal lung, kidney, and skeletal muscle but not in the corresponding adult tissues, suggesting that caspase 10 might also play an important role in fetal development.

In our present study, we found significantly lower expression of caspase 8 and 10 mRNA and/or protein in CCA and HM compared with non-molar placentas. Our results support the hypothesis that human cancer cells often have defects in apoptosis, including deficient expression of caspases. For example, downregulation of caspase 1 or 7 appears to be a marker of colonic cancer. In contrast, caspase overexpression may induce apoptosis in a prostate cancer cell line. Thus, the significant downregulation of caspases 8 and 10 detected in our study may play a role in the pathogenesis of GTD. However, no significant differences in the expression of caspases 8 and 10 were seen between regressive and persistent moles, suggesting that the expression of these caspases would not be a useful predictor of the clinical behaviour of HMs.

"Our results support the hypothesis that human cancer cells often have defects in apoptosis, including deficient expression of caspases"

Trophoblasts of the placenta can be considered to be "pseudomalignant" tissue because even normal trophoblasts display features of malignant cells, such as rapid proliferation, infiltration of host tissue, and haematogenous dissemination, and can escape immunological surveillance. Trophoblasts can be divided into CTs, STs, and intermediate trophoblasts. CTs consist of proliferating stem cells and subsequently differentiate and fuse to form STs. It has been found that different oncogenes are expressed differentially in CTs and STs. Our immunohistochemical staining analysis showed that caspase 8 expression was limited to CTs, whereas caspase 10 was expressed predominantly in STs.

With no generative potential, the formation of an ST depends upon the continuous fusion of the underlying proliferating CT to replace its aging population. This continuous fusion of stem cells also serves to transport fresh cellular materials such as nucleic acids and enzymes, which might include caspases, to the ST. In culture conditions, it was shown that CTs exhibit higher caspase 3, 6, 8, and 9 activity compared with the more differentiated STs, and that caspase 8 expression is a prerequisite for differentiation and syncytial fusion in CTs. Because caspase 10 was only weakly expressed in CTs but strongly expressed in STs, caspase 10 activity may start in the CT and accumulate during syncytial fusion at ST. Caspase 8 expression was found in CT only; it might become active in the CT but disappear once syncytial fusion is completed. Our previous findings from the M30 study also suggested that apoptosis involving the caspase cascade might be initiated in the CT and completed in the ST. These data appear to support the view that caspase 8 occupies an upstream position with respect to caspase 10 in the apoptotic pathway in trophoblasts. Alternatively, caspases 8 and 10 may have distinct roles in death receptor signalling in apoptosis initiation in different populations of trophoblasts.

The immunoreactivity of both caspase 8 and caspase 10 correlated with the apoptotic index in GTD assessed by the M30 and TUNEL approaches in our previous studies. The TUNEL technique identifies fragmented DNA formed as a result of DNA endonuclease activity and helps in assessing apoptosis at a late stage. The M30 monoclonal antibody recognises a formalin resistant caspase cleavage site within cytokeratin 18, after cleavage by caspases 3 and 7. A significant correlation between caspases 8 and 10 and both of these apoptotic activity indicators suggested that caspases 8 and 10 are important in the process of apoptosis. In contrast, neither caspases 8 nor caspase 10 expression correlated with that of Bcl-2, suggesting that the expression of Bcl-2 does not influence the expression of caspases 8 and 10 in GTD. Therefore, further studies to elucidate the relation between Bcl-2 and specific caspases are essential.

**Take home messages**

- We found significantly lower expression of caspase 8 and 10 mRNA and/or protein in choriocarcinoma and hydatidiform mole compared with non-molar placentas.
- Immunoreactivity for both caspase 8 and caspase 10 correlated with the apoptotic index.
- Caspase 10 and its related family member caspase 8 are probably important in the regulation of apoptosis in trophoblastic tissues and the development of trophoblastic diseases.
- Apoptosis involving the caspase cascade might be initiated in the cytotrophoblast and completed in the chorionic villi of the syncytiotrophoblast.
In summary, caspase 10 and its related family member caspase 8 are probably important in the regulation of apoptosis in trophoblastic tissues and the development of GTD. Apoptosis involving the caspase cascade might be initiated in the CT and completed in the chorionic villi of the ST stage.

ACKNOWLEDGEMENTS

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