ORIGINAL ARTICLE

Nuclear β -catenin and Ki-67 expression in choriocarcinoma and its pre-malignant form

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Objective: To study the expression of nuclear β -catenin and Ki-67 in patients with normal gestation products (NGP), complete hydatidiform moles (CHM), and choriocarcinoma to elucidate their roles in carcinogenesis and their interrelations.

Methods: Expression of nuclear β -catenin and Ki-67 was studied by immunohistochemistry using paraffin embedded blocks. Sixty NGP, 60 CHM, and 10 choriocarcinomas were analysed. In addition, approximately 400 trophoblasts each in 40 NGP, 40 CHM, and 10 choriocarcinomas from the same batch of samples were microdissected for quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) to compare β -catenin mRNA concentration among them.

Results: In the chorionic villi of NGP, β -catenin was consistently expressed in the nuclei of cytotrophoblasts but not syncytiotrophoblasts. Nuclear β -catenin expression was comparatively reduced in CHM trophoblasts and was absent in choriocarcinoma. By contrast, Ki-67 expression was increased from cytotrophoblasts but not in syncytiotrophoblasts in the chorionic villi of NGP to CHM trophoblasts and choriocarcinoma. Using Q-RT-PCR, β -catenin mRNA was detected in 10 NGP, 13 CHM, and three choriocarcinoma specimens, with median copy numbers of 43 230, 18 229, and 17 334 per 400 trophoblasts, respectively. A housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected as a control in the NGP, CHM, and choriocarcinoma specimens, with median copy numbers of 51 300, 54 270, and 97 150 per 400 trophoblasts, respectively. Thus median β -catenin mRNA values after normalisation were 0.85 in NGP (n=10), 0.31 in CHM (n=13), and 0.16 in choriocarcinoma (n=3).

Conclusions: Decreased nuclear β -catenin expression and increased Ki-67 expression may be involved in choriocarcinoma carcinogenesis. The findings also suggest that nuclear β -catenin may play a role in trophoblast differentiation during normal placental development.

Choriocarcinoma is a highly malignant neoplasm of trophoblastic cells arising from gestational trophoblastic disease, especially complete and partial hydatidiform moles (CHM and PHM).¹ The incidence of hydatidiform mole is 1–3 per 1000 pregnancies² and between 8% and 30% will develop into choriocarcinoma. Choriocarcinoma is normally treated with chemotherapy and is monitored by measuring the serum β -human chorionic gonadotropin (β -hCG) concentration.³ Although various biological markers have been found—including c-erbB-2,⁴ c-ras,⁴ nm23,⁴ p53,⁴ DOC-2/hDab2,⁵ cyclin E,⁶ telomerase activity,⁷ apoptotic index,⁸ matrix metalloproteinases,⁹ Ras GTPase activating protein,¹⁰ and hypermethylation of p16³—the key biological pathway is still unknown and recent studies have shown the involvement of β -catenin, a key target in the Wnt-signalling pathway, in trophoblast differentiation.^{11–13}

In this study we investigated the expression of β -catenin in patients with normal gestation products (NGP), CHM, and choriocarcinoma to elucidate their roles in carcinogenesis. In addition, the proliferation index, Ki-67, was also measured in order to evaluate the interplay of β -catenin with proliferation and elucidate their possible interrelation in this pathway. Finally, β -catenin mRNA concentration was measured using approximately 400 cytotrophoblasts per paraffin embedded section each from 40 NGP, 40 CHM, and 10 choriocarcinomas for quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) after microdissection.

RNA quantitation in formalin fixed, paraffin embedded tissue has two major drawbacks. One is extensive degradation of RNA before completion of the formalin fixation process¹⁴ and the other is that formalin fixation can cause

cross linkage between nucleic acids and proteins which may covalently modify RNA by the addition of monomethylol groups to the bases.¹⁵ In order to ensure reliable RNA extraction, reverse transcription and quantitation, we also measured the concentration of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, a housekeeping gene,¹⁶ for normalisation.

The information gathered from this study may aid in understanding the biology of human trophoblastic diseases. In the long run, it may contribute towards the development of prognostic markers and targeted treatment aimed at restoring the defects of this pathway in affected tumour cells.

METHODS

The study was approved by the local institutional research ethics committee.

Tissues

Formalin fixed, paraffin embedded specimens of 60 NGP, 60 CHM, and 10 choriocarcinomas (1985 to 2004) were retrieved from the archives of the department of pathology, Queen Elizabeth Hospital, Hong Kong, for immunohistochemical staining. From the same batch of specimens, 40 NGP, 40 CHM, and 10 choriocarcinomas were also recruited for Q-RT-PCR.

Abbreviations: β -hCG, β -human chorionic gonadotropin; CHM, complete hydatidiform mole; DEPC, diethylpyrocarbonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; NGP, normal gestation products; PHM, partial hydatidiform mole

Table 1 Sequences of primers, probe, and calibration oligonucleotide

<i>β-catenin</i> mRNA			
Intron spanning primers	Forward	Exon 2	5'- GCGTGGACAATGGCTACTCA-3'
	Reverse	Exon 3	5'- CCGCTTTTCTGTCTGGTTC-3'
Dual labelled fluorescent probe		Exon 3	5'- (FAM [®])TGATTGATGGAGTTGGACATGGCCA (TAMRA [®])-3'
Oligonucleotide for calibration			5'-ATCCAGCGTGGACAATGGCTACTCAAGCTGATTGATGGAGTTGGACATGGCC ATGGAACACAGACAGAAAAGCGCTGT-3'
<i>GAPDH</i> mRNA			
Intron spanning primers	Forward	Exon 2	5'-GAAGGTGAAGGTGGAGT-3'
	Reverse	Exon 4	5'-GAAGATGGTGATGGGATTC-3'
Dual labelled fluorescent probe		Exon 4	5'-(FAM [®])CAAGCTTCCCCTTCTCAGCC(TAMRA [®])-3'

φ, 6-carboxyfluorescein.

♦, 6-carboxytetramethylrhodamine.

Antibodies

β-Catenin monoclonal antibody (C19220, Transduction Laboratories, Lexington, Kentucky, USA) and Ki-67 monoclonal antibody (Clone MIB-1, M7240, DakoCytomation, Glostrup, Denmark) were used.

Immunohistochemical staining and evaluation

Serial tissue sections (4 μm thick) were cut and antigen retrieval was achieved by heating the sections in EDTA solution, pH 8.0, in a pressure cooker for 2.5 minutes. Staining was undertaken in a Ventana-NexES automated immunostainer (Tucson, Arizona, USA) at 37°C using 1/500 dilution of *β*-catenin antibody and 1/200 dilution of Ki-67 antibody, with a labelled streptavidin-biotin peroxidase system (I-view kit, Ventana, Tucson, Arizona). A colon carcinoma sample was mounted on every test slide as a positive control, and negative controls were achieved by replacing the antibody with Tris buffered saline. Positive staining was localised in the nuclei (*β*-catenin and Ki-67) and membrane (*β*-catenin). The slides were evaluated in five fields under light microscopy at 400× magnification. Cytoplasmic staining was not assessed, as endogenous biotin may be picked up by the detection system. As the cellularity and cell density of choriocarcinoma cases varied significantly, we carefully selected tumour areas so that at least 1200 cells in five fields were counted and there was 100% cellularity without any obstacle per field, respectively, for microscopic examination and scoring. All positive cases were scored semiquantitatively and expressed as an immunohistochemistry (IHC) score by multiplying the "percentage of positive cells" by the "staining intensity", as described previously.^{17, 18} Staining intensity was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong; 4, very strong. The IHC score ranged from 0 to 400.

RNA extraction and reverse transcription reaction

All formalin fixed, paraffin embedded blocks were cut in 2×8 μm thick sections, stained in haematoxylin and eosin solution, and microdissected under a microscope using sharp needles. Approximately 400 trophoblasts—chorionic villi in NGP, CHM trophoblasts, and choriocarcinoma cells—were microdissected per section and put into RNase-free tubes for extraction, which began by adding 1.6 ml Trizol reagent (Life Technologies, Carlsbad, California, USA), 0.4 ml chloroform before centrifugation, and further purified with an RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions and dissolved in 30 μl of diethylpyrocabonate (DEPC) treated water. Reverse transcription was undertaken using the TaqMan Gold RT-PCR kit (Applied Biosystems: N808-0233, Foster City, California, USA), following the recommended protocol after DNase I treatment (Life Technologies). cDNA was generated in a 20 μl reaction mix and stored at -20°C until use. Each batch of reaction included a positive control for *β-catenin* mRNA and negative controls without RNA and reverse transcriptase.

Q-RT-PCR assay for *β-catenin* mRNA and *GAPDH* mRNA

Intron panning primers and fluorescent probes were designed for *β-catenin* detection. For *GAPDH* mRNA detection, the primers and probe were purchased from Applied Biosystems (table 1). Q-RT-PCR was set up in a reaction volume of 50 μl using Core Reagents kit (Applied Biosystems), and 8 μl of cDNA was used for each reaction. The standard protocol of the ABI Prism 7000 sequence detector (Applied Biosystems) was used in the Q-RT-PCR assay. A calibration curve for *β-catenin* mRNA quantification was prepared by amplifying serial dilutions of a synthetic oligonucleotide (table 1), with concentrations ranging from 2.5×10⁶ to 25 copies/μl. Similarly, the *GAPDH* mRNA calibration curve was prepared by serial dilutions of human control cDNA (Applied Biosystems), with concentrations ranging from 2.6×10⁵ to 41 copies/μl based on manufacturer's information that 1 pg of this control RNA contained approximately 100 copies of *GAPDH* transcripts. Each batch of amplification included a positive control with *β-catenin* mRNA and *GAPDH* mRNA, and a negative control without cDNA. Duplicate tests were done and the average was calculated for each sample. Finally, a normalised value for *β-catenin* mRNA in each sample was obtained by dividing absolute *β-catenin* mRNA concentration with its corresponding *GAPDH* mRNA concentration.

Statistics

The relations between nuclear *β*-catenin IHC scores and the pathological stages of choriocarcinoma and Ki-67 expression were studied using the Kruskal-Wallis test and Spearman rank correlation, respectively. GraphPad Prism software version 4.0 (GraphPad, Software Inc, San Diego, California, USA) was used for all statistical analyses, and p<0.05 was considered significant.

RESULTS

Immunohistochemical staining

β-Catenin

β-Catenin was consistently expressed in both the nuclei and the membranes of cytotrophoblasts but not in the syncytiotrophoblasts in the NGP chorionic villi (fig 1A). In CHM, *β*-catenin expression was decreased in the nuclei and membranes of cytotrophoblasts when compared with those in NGP (fig 1B). The nuclear *β*-catenin signal was also found in some trophoblasts with no nuclear pleomorphism within small, early-forming molar tissues (fig 1B), whereas only membrane and no nuclear *β*-catenin was found in trophoblasts with nuclear pleomorphism within large, mature molar tissues (fig 1C). In choriocarcinoma, a majority of the tumour cells expressed only membrane *β*-catenin and a minority did not express any *β*-catenin at all (fig 1D).

Figure 1 Immunostaining for β-catenin and Ki-67 antibodies in the same area. (A) Normal gestation products (NGP) with β-catenin expressed in the nuclei and membranes of cytotrophoblasts. Inset: a higher ($\times 600$) magnification shows expression of β-catenin in the nuclei of cytotrophoblasts. (B) β-Catenin expressed both in the nuclei and membranes of cytotrophoblasts in a complete hydatidiform mole (CHM) without nuclear pleomorphism. (C) β-Catenin expressed in the membranes of cytotrophoblasts in a CHM with nuclear pleomorphism. (D) β-Catenin expressed in the membrane of choriocarcinoma cells. Inset: choriocarcinoma cells without β-catenin expression. (E) Positive control: β-catenin expressed in the nuclei and membranes of a colon carcinoma. (F) Negative control in an NGP. (G) NGP with Ki-67 expressed in the nuclei of cytotrophoblasts. (H) Ki-67 expressed in the nuclei of cytotrophoblasts in a CHM without nuclear pleomorphism. (I) Ki-67 expressed in the nuclei of cytotrophoblasts in a CHM with nuclear pleomorphism. (J) Choriocarcinoma with Ki-67 expressed in the nuclei of tumour cells. (K) Positive control: Ki-67 expressed in the nuclei of a colon carcinoma. C, cytotrophoblasts; S, syncytiotrophoblasts; T, tumour; Tr, trophoblasts. Original magnification, 400 $\times$.

Figure 2 Immunohistochemistry (IHC) scores in 60 normal gestation products (NGP), 60 complete hydatidiform moles (CHM), and 10 choriocarcinoma cases. (A) Nuclear β -catenin; (B) Ki-67. The median in each group of subjects is indicated by a horizontal line.

Ki-67

Expression of Ki-67 was found in the nucleus. In contrast to β -catenin, Ki-67 had a low expression in the cytotrophoblasts and was not expressed at all in syncytiotrophoblasts in the NGP chorionic villi (fig 1G). A moderate expression was found in cytotrophoblasts in the chorionic villi and in trophoblasts in CHM (fig 1H). The highest Ki-67 expression was observed in choriocarcinoma (fig 1J). In contrast to β -catenin, Ki-67 positivity increased from the trophoblasts with no nuclear pleomorphism (fig 1H) to those with nuclear pleomorphism in CHM specimens (fig 1I).

The IHC scores for nuclear β -catenin and Ki-67 expression for all specimens at each clinical stage are shown in fig 2, panels A and B, respectively. The decrease of β -catenin and the increase of Ki-67 expression were correlated with clinical stage (Kruskal-Wallis test, $p < 0.05$ for both β -catenin and Ki-67). The negative correlation of β -catenin with Ki-67 expression was also highly significant (Spearman rank correlation, $p < 0.05$). All positive controls showed intense β -catenin (nuclear and membranous) expression (fig 1E) and Ki-67 nuclear expression in colon carcinoma cells (fig 1K). No signal was observed in the negative controls (fig 1F).

Q-RT-PCR assay for β -catenin mRNA and GAPDH mRNA

The calibration curve was not extrapolated, so all positive results were within the range detected by the calibration standards in each Q-RT-PCR assay. All positive controls had expected quantities of β -catenin mRNA and GAPDH mRNA. Negative controls did not have any signal in each batch of assay. β -Catenin mRNA was positive in 10 of 40 NGP (25%), 13 of 40 CHM (33%), and 3 of 10 choriocarcinoma (30%), with median copy numbers of 43 230, 18 229, and 17 334 per 400 trophoblasts, respectively (fig 3). Moreover, GAPDH mRNA was positive in the same 10 of 40 NGP (25%), 13 of 40 CHM (33%), and 3 of 10 choriocarcinoma (30%), with median copy numbers of 51 300, 54 270, and 97 150 per 400 trophoblasts, respectively (fig 4). Therefore, normalised

Figure 3 Absolute copy numbers of β -catenin mRNA in 10 normal gestation products (NGP), 13 complete hydatidiform moles (CHM), and three choriocarcinoma cases. The median in each group of subjects is indicated by a horizontal line.

values of β -catenin mRNA ranged from 0.56 to 0.94 (median = 0.85) in NGP ($n = 10$), from 0.14 to 0.80 (median = 0.31) in CHM ($n = 13$), and from 0.12 to 0.18 (median = 0.16) in choriocarcinoma ($n = 3$) (fig 5). None of the other samples (30 NGP, 27 CHM, 7 choriocarcinomas) had any β -catenin mRNA or GAPDH mRNA and they were therefore scored as amplification failure.

DISCUSSION

The biology of the trophoblast is interesting as some aspects of the trophoblast are similar to those of a malignant cell,¹⁹ especially with regard to proliferative and invasive properties.²⁰ Approximately 50% of the choriocarcinoma cases are preceded by CHM and only 20% of cases arise from normal pregnancies.²¹ Choriocarcinoma has both invasive and metastatic properties and there are occasions of fatal cases in which metastases are found in the lungs, brain, bone marrow, and liver.¹⁹ Therefore an improved understanding of the biology of its pathogenesis would be helpful in the development of new markers for early detection.

β -Catenin is a key regulator in the cadherin mediated cell adhesion system. Its expression in the nucleus is a potential marker in colorectal cancer.¹⁷ Our immunohistochemical findings of decreased nuclear β -catenin expression in cytotrophoblasts from NGP to trophoblasts in CHM and choriocarcinoma reveal, for the first time, the involvement of nuclear β -catenin in the oncogenesis of choriocarcinoma. Our results reaffirm a previous report that showed an important role of Wnt signalling in human fetal development with nuclear β -catenin in cytotrophoblasts.²² In addition, the differential expression of nuclear β -catenin in early-forming molar tissue but not in their mature form suggests that nuclear β -catenin may have a role in the maturation of CHM. In addition, our immunohistochemical findings were also confirmed by the Q-RT-PCR, showing that there was a significant decrease in β -catenin mRNA concentration from cytotrophoblasts in NGP to trophoblasts in CHM to

Figure 4 Absolute copy numbers of GAPDH mRNA in 10 normal gestation products (NGP), 13 complete hydatidiform moles (CHM), and three choriocarcinoma cases. The median in each group of subjects is indicated by a horizontal line.

choriocarcinomas. In combination with genomics, these paraffin embedded specimens represent an invaluable resource for the elucidation of disease mechanisms and validation of differentially expressed genes as novel therapeutic targets or prognostic indicators. However, the application of this technology in paraffin embedded tissue has a major limitation of an inhibitory effect of formalin fixation^{23, 24} and paraffin embedding²³ on RNA quality that requires the use of a housekeeping gene such as *GAPDH* mRNA as an internal reference standard to ensure reliable and consistent mRNA quantitation. Our results showed a 2.5-fold decrease and a 1.9-fold increase in β -catenin mRNA and *GAPDH* mRNA, respectively, from NGP to choriocarcinoma. We speculate that the increase in *GAPDH* mRNA may not only reflect variations in fixation, paraffin embedding, and technical skills in mRNA extraction interfering with the integrity of the RNA. They may also indicate that choriocarcinoma cells actually have more diverse cellular functions than cytotrophoblasts in NGP and trophoblasts in CHM, based on previous studies that the *GAPDH* gene is also involved in many diverse cellular functions unrelated to glycolysis. These include nuclear RNA export, DNA replication, DNA repair, exocytotic membrane fusion, apoptosis, neurodegenerative disease, prostate cancer, and viral pathogenesis.^{25, 26}

Irrespective of these variations, normalised β -catenin mRNA concentration decreases fivefold from NGP to choriocarcinoma. In CHM, β -catenin mRNA concentration was similar to that of choriocarcinoma whereas its *GAPDH* mRNA concentration was similar to that of NGP. We can draw two conclusions from these findings. First, β -catenin mRNA quantitation actually matches its immunohistochemical results because β -catenin is mainly expressed in the membrane of trophoblasts in both CHM and choriocarcinoma. Second, *GAPDH* mRNA quantitation can provide evidence that the biological properties of CHM are similar to those in NGP, and this is logical because CHM is only a premalignant form of an invasive mole or a choriocarcinoma. However, the low sensitivity of Q-RT-PCR emphasises the need for further improvements in the fixation and processing of paraffin embedded specimens. Despite this, normalised values of β -catenin mRNA can still show that there were distinct differences at all clinical stages. On the other hand, our immunohistochemical finding of nuclear β -catenin expression in the cytotrophoblasts of NGP contrasted with that of Li *et al*²⁷ and Getsios *et al*,¹³ who reported that β -catenin was expressed in the cytoplasm and membrane but not in the nucleus. The explanation for this variation may be that Li *et al* used a milder microwave based antigen retrieval method and Getsios *et al* used frozen section for β -catenin

Figure 5 β -catenin mRNA copy numbers in 10 normal gestation products (NGP), 13 complete hydatidiform moles (CHM), and three choriocarcinoma cases after normalisation with their respective *GAPDH* mRNA copy numbers. The median in each group of subjects is indicated by a black horizontal line.

immunostaining, involving 4% paraformaldehyde fixation, which may be less efficient in preserving antigens inside the nucleus. The significant association of a decreased nuclear β -catenin expression in choriocarcinoma carcinogenesis—in contrast to our previous findings of an increased β -catenin expression in colorectal carcinogenesis¹⁷—reflects the fact that nuclear β -catenin may be involved in the development of placental cytotrophoblasts, and stimuli other than nuclear β -catenin may be required for the cytotrophoblast to evolve into CHM and choriocarcinoma. Further studies are warranted to explore the function of nuclear β -catenin in benign cytotrophoblasts.

Ki-67, an antigen present in G1, S, G2, and M phases but absent in the G0 phase of the cell cycle, appears to be an excellent indicator of tumour proliferation.²⁸ Our data indicate a continuous increase in proliferation from cytotrophoblasts in NGP to CHM to choriocarcinoma, in line with the results of Uzunlar *et al*,²⁹ who showed that proliferation was significantly greater in gestational trophoblastic diseases than in NGP from spontaneous abortion. An observation worthy of attention is that there are many cytotrophoblasts in the NGP with nuclear β -catenin that do not express Ki-67 antigen. We hypothesise that nuclear β -catenin may play an important role in cell cycle arrest. In addition, the negative correlation of nuclear β -catenin to Ki-67 expression suggests that loss of nuclear β -catenin may account for the proliferation in the oncogenesis of choriocarcinoma.

It is well known that CHM with clinical or histopathological evidence of excessive abnormal activity is more likely to develop into an invasive mole or even a choriocarcinoma. Thus patients with such CHM should be considered for prophylactic chemotherapy.³⁰ Decreased β -catenin and increased Ki-67 expression in the nuclei of the trophoblasts with different maturity in CHM, as highlighted in the present study, may provide an explanation for this phenomenon. In fact, hCG immunostaining indicates that most of the trophoblasts in CHM were hCG negative (data not shown), which shows that the majority of trophoblasts in CHM are derived from cytotrophoblasts. This finding is compatible with our observations that nuclear β -catenin in cytotrophoblasts of NGP become proliferative in order to develop into CHM, with decreasing β -catenin expression from nucleus to membrane. Although decreased nuclear β -catenin expression and increased Ki-67 expression may be involved in choriocarcinoma carcinogenesis, those findings still have to be confirmed by other in-depth investigations, as there are only 10 choriocarcinoma cases in our archives. This reflects the fact that this disease now responds very well to chemotherapy.

Conclusions

This study has provided new information on the biology of choriocarcinoma formation, and has shown that decreased nuclear β -catenin and increased Ki-67 expression are features of choriocarcinoma formation. These results also highlight the possibility that nuclear β -catenin may play a role in trophoblast differentiation during normal placental development. Further investigation into the role of nuclear β -catenin in choriocarcinoma or its premalignant form may be beneficial for high risk molar pregnancy patients, as choriocarcinoma can have a latent period of more than 15 years after evacuation of the molar tissues.

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