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RESEARCH

Altered p16 and Bcl-2 Expression Reflects Pathologic Development in Hydatidiform Moles and Choriocarcinoma

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Abstract Abnormal trophoblast differentiation is the main cause of gestational trophoblast diseases in the case of hydatidiform moles and choriocarcinomas. Here we investigated the expression patterns of two gene products, p16 and Bcl-2, implicated in cell cycle regulation and apoptosis, respectively, using immunohistochemistry during normal placenta differentiation, hydatidiform moles (partial, complete and invasive) and post-molar choriocarcinomas. The p16 protein shows a gradual expression in cytotrophoblast of normal villous, from a p16 weak proliferative phenotype to a p16 strong invasive phenotype reaching a maximum around 17 weeks of gestation. The expression pattern in cytotrophoblast was similar in moles in contrast to the villous mesenchyme of invasive moles where p16 was strongly expressed. Bcl-2 expression was syncytiotrophoblast specific in normal placenta and moles and increased gradually during normal differentiation. The results explain the persistence of normal and molar villous fragments during their development and their dramatic invasion in the uterine arteries in case of invasive moles. In choriocarcinomas the weak Bcl-2 expression is associated with weak p16 expression indicating a great apoptotic and proliferative potentials. The results suggest that strong p16 expression in the villous mesenchyme may be responsible in part of the morbidity of the moles, and the key of cancer progression in the choriocarcinomas would be a fast cell-cycle turnover.

Keywords Trophoblast · Uterine cancer · Differentiation · p16 · Bcl-2

Abbreviations

CTB	Cytotrophoblast
STB	Syncytiotrophoblast

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Summary sentence: p16 and Bcl-2 are important actors during trophoblast invasion of endometrium and/or maternal tissues in the cases of invasive hydatidiform moles and choriocarcinoma

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EVT Extravillous trophoblast
CHM Complete hydatidiform mole
PHM Partial hydatidiform mole

Introduction

Trophoblast differentiation is directly related to the placental epithelial stem cells, termed cytotrophoblast (CTB), which always reside in tight contact with the basement membrane. CTB stem cells differentiate by fusing to form multinucleated syncytiotrophoblast (STB). Ultimately, the overlying syncytium establishes a contact directly with the maternal blood. Extravillous trophoblast (EVT) cells derive from CTB by proliferation and differentiation as they leave the basement membrane and form cellular aggregates. The human placenta is formed by extensive interstitial (endometrium) and vascular (spiral artery) invasion of EVT in the maternal uterus. This invasive process have to be limited (veto) to avoid an uterine pathology [1]. Several factors intervene to establish a delicate balance between cell proliferation, differentiation and apoptosis [2, 3], among them cell cycle regulators and anti-apoptotic molecules play dominant roles during these key transitions [4]. Differentiation of invasive CTB is coordinated with their progressive exit from the cell cycle. To better understand the mechanisms of this transition we selected two stable markers used in histopathology, the cell cycle regulator p16 and the apoptosis inhibitory Bcl-2.

p16(INK4a) (cyclin dependent kinase inhibitor) is a tumor suppressor protein, encoded by the *CDKN2A* gene located on chromosome 9p21 in human [5]. The p16(INK4a) product is a nuclear protein which regulate cell proliferation mainly by down-regulating cyclin-dependent kinases (CDKs) 4 and 6 activity and thus leading to an arrest in G_0/G_1 cell-cycle checkpoint. Deletion of the *CDKN2A* gene has been identified in most human tumors [6, 7] and in endometrial carcinoma it was associated with increased incidence of metastases [8]. p16 (INK4a) was also implied in CTB differentiation [4] in invasion of trophoblastic cells, during the process of blastocyst implantation in mice [6] and at some distance from trophoblast cell columns [1]. Deletion of the *CDKN2A* area was identified in two of 12 post-molar choriocarcinoma cases, studied by comparative genomic hybridization [9]. Moreover, during the molar process and neoplastic transformation of trophoblast, *CDKN2A* has been found globally hypermethylated as compared to the normal placenta. This status significantly correlated with a reduced expression of this gene [10] that could result in subsequent development of gestational trophoblastic neoplasia.

Invasion of EVT toward the endometrium is precisely regulated, where apoptosis could play a role, mainly during the first trimester of placental development. The incidence of apoptosis was particularly visible in the syncytial layer [11]. Importantly, Kim et al. [12] have shown that expression of the anti-apoptotic Bcl-2 protein (B-cell leukaemia/lymphoma 2) decreased in the placenta during the third trimester, indicating for increased tissue remodelling involving apoptosis. In addition, initiation of nuclear degeneration in STB progresses in parallel with CTB fusion. This apoptotic cascade is initiated in the villous CTB which in turn promotes syncytial fusion [13]. However, Bcl-2 expression is finely and differentially regulated during this first trimester which is essential for the maintenance of normal pregnancy [14–16]. In EVT cell columns Bcl-2 expression was pronounced only in the surface layer (proliferative phenotype) while apoptosis was prominent in the deeper region (invasive phenotype). Bcl-2 participates in the regulation of apoptosis in EVT along invasion of the decidua. The increased frequency of apoptosis in the invasive EVT phenotype may be attributed to a decreased Bcl-2 expression in term placenta [17, 18]. Bcl-2 gene is located on chromosome 18 q21.3 [19, 20]. Altered expression of this protein occurs commonly in human cancers, contributing to neoplastic cell expansion by veto apoptosis and extending tumor cell life span [21].

Hydatidiform moles and choriocarcinomas are two major subtypes of gestation trophoblastic diseases, and about 50 % of the choriocarcinomas are post molar but most choriocarcinoma are related to previous complete hydatidiform moles (CHM) and more rarely to partial hydatidiform moles (PHM) [22]. During the molar differentiation, another neoplastic derivative can occur, i.e. the invasive mole (invHM). Typically they cause invasion of villous fragments into the uterine myometrium (invasive mole) or cause metastasis in distant organs via the blood stream (choriocarcinoma). The molar conceptus is characterized by hydropic (liquid-filled) enlarged chorionic villi [23]. We can identify the PHM by the presence of various embryonic traces, amniotic sac or vasculature. Generally, their karyotype is triploid with one maternal set and two paternal sets of chromosomes [24, 25]. These conceptuses originate from an ovocyte fertilized by a diploid spermatozoid or more frequently with two spermatozoids (dispermia) [26]. The CHM shows generalized hydropic degeneration extended to the entire villosity of the placenta with any traces of embryo. The karyotype is generally diploid with 46 XX (90 %) and all the chromosomes are of paternal origin. In 80 % of the cases, CHMs are homozygous, and interestingly the majority of CHMs may share an androgenic origin [27, 28]. They are usually sporadic and their incidence varies among countries. In western countries their frequency is approximately 0.5–1 per 1000 conceptus, while is highest in South-East-Asia with rates ranging from 1 to 2/1000 pregnancies in Japan and China and 12/1000 pregnancies in Indonesia, India and Turkey [29]. In the cases of CHM these data decreased recently due to the improvement of socio-economic and nutritional conditions [30, 31]. The initial defect of these pathologies

may be associated with imbalanced imprinting of genes which were complete in the cases of androgenic CHM [32]. In 10 % of PHM and CHM the diploid karyotype was found normal, biparental. PHM and CHM may result from mutation of the NLRP7 gene [33] a NALP family member implicated in inflammatory processes by activating proinflammatory caspases via multimolecular inflammasome complexes. The deleterious effect of NLRP7 mutation may occur during ovogenesis, fertilization or embryonic development. Our knowledge on the precise cellular and molecular mechanisms of these pathologies, however, still remained incomplete.

To get a better insight in the role of p16 and Bcl-2 gene products in hydatidiform moles and choriocarcinoma here we investigated the expression patterns of these proteins through development of healthy and diseased conceptuses.

Materials and Methods

Patients and Samples

Our studies were conducted on a panel of selected representative normal placenta and pathologic samples including different kinds of moles and choriocarcinomas. Permission for the study was granted from the French national ethics committee. Aborted embryos and foetuses were obtained in accordance with French guidelines. Ages were estimated according to specific anatomical markers and were given as gestational age (from the fertilization to childbirth).

The main characteristics of these samples were reported in Table 1.

Normal Placenta

Control tissue samples were obtained from legal abortions at 8/10 weeks gestational stages (WGS), from therapeutic abortion at 15/18 WGS and from childbirth at 37/39 WGS corresponding to three developmental stages. Two cases of twin pregnancies consisting of hydatidiform moles M246 and M253 and normal fetuses respectively M247 and M254, allowed the normal placentas and moles to compare at the same ages.

Pathologic Samples

Specimens of trophoblastic tissues were collected at different departments of pathology in France and Africa. Among our collection, twenty-five cases were selected for this study accordingly to the etiology and the integrity of molar tissues. As shown (Table 1), we differentiated 8 PHM, 14 CHM, 5 invasive hydatidiform moles (invHM) and 8 post molar choriocarcinomas. The deciduas were also considered for all normal samples and for 8 pathologic samples. Considering our selection criterions (see below), and the great difficulties (tissues quality, exotic pathologies and ethics) to obtain all the types of the different molar pathologies (particularly biparental moles) we did not the possibility to increase the number of samples.

Table 1 Normal placenta (italic) and pathological samples

Names	Types	caduc	Genomic analyses	Cytogenetic analyses	Geographical Origin
8/10, 15/18, 37/39	*Normal placenta*	+	–	–	France
5507, M160, M219	PHM	−	3n	–	France
5929, 4482	PHM	+	3n	–	France
M251	biPHM	−	2n	NLRP7; XY	France
M264-1, 264-2	biPHM	+	2n	NLRP7; (1)XX; (2)XY	France
M104, 141, 144, 246, 253	anCHM	−	2n	M141: XX	France
M 112, 113, 114, 155, 156, 158	CHM	−	–	–	France
					Benin
213					Algeria
M246/247, M253/254	anCHM/normal Twin	−	2n (dizygote)	M253: XX	France
				M254: XY	
M103, 142	Inv HM	−	2n anCHM	M142: XX	France
5034, 7278, 9746	Inv HM	+	–	–	France
M131, 165, 170, 176, 181, 232, 235	Choriocarcinoma	−	2n anCHM	–	Senegal
3911	Infiltrant choriocarcinoma	+	–	–	France

Ages of normal Placenta was given in weeks from fertilization to abortion or term. 3 placental tissues at each 3 stages were analysed. M247 and M254 were placental samples of dizygote normal twins of respectively CHM M246 and M253. NLRP7: the patient is deficient for her NLRP7 gene. M264-1 and M264-2 were the successive PHM of the same genitors

Inclusion Diagnostic and Confirmation

All the pathological cases included in this study correspond to standard clinical criterions and confirmed by anatomopathologists: biochemical proportioning of betaHCG, immunohistochemistry (especially the expression of $p57^{kip2}$ imprinted gene which was maternally expressed) and histological descriptions. Some patients profited from a tracking or from a scan diagnosis. These studies were performed according the 2000 FIGO scoring system (FIGO: International Federation of Gynecology and Obstetrics) [34].

Complementary Genetic Analysis and Informations

As mentioned Table 1 some of our samples (mainly those obtained not fixed during our prospective studies) could beneficiate of genetic analyses completing their characteristics about their etiology and cytogenetic patterns.

Genotyping Owing their DNA and each time it was possible also from the patient or from the two genitors, and using a semi quantitative method [35] and a set of microsatellites, genotyping could be realised providing indications and confirmation about the etiology of pathological samples. So, M251 could be assigned of bi-parental origin (biPHM), M103, M104, M141, M142, M144, M246 and M253 from androgenic monospermic origin (anCHM), and particularly the choriocarcinomas M131, M165, M170, M176, M181, M232, M235 were confirmed as derived from androgenetic monospermic moles by genotyping analyze. In addition the two couples of molar and normal twins were confirmed as dizygote.

Cytogenetics Full caryotype were realized on cell primary cultures on M141 (46 XX), M142 (46XX), M 251 (46XY), M253 (46XX) and M254 (46XY). When the cell primary culture was not possible (the most common case), the ploïdy was controlled with FISH probes X and Y.

Cibled in situ hybridization on histological slides allowed to precise that M264-1 and M264-2 were diploid and respectively XX and XY. Chromosome gains and looses were studied by array CGH high resolution 244K on choriocarcinomas M165, M170, M176, M181, M232 and M235.

NLRP 7 Sequencing NLRP7 mutations were documented for the 2 patients implicated for 2n PHM M251 and M264-1 &2 (Dr. R. Slim personal communication). In the case of other hydatidiform moles (androgenic ones) we have not observed any recurrent moles in the family. We can postulated that the mutation NLRP7 must not be the cause of these pathologies [33].

In addition some of these cases had been previously published for other studies: M 251 as patient 662 in Deveault et al. [33] and M141, M253, M254 in Noguer-Dance et al. [36].

Immunohistochemistry

Tissue samples were fixed using 10 % formalin, dehydrated and embedded into paraffin. Sections 3 μm thick were cut and mounted on superfrost plus slides, stained with hematoxylin and eosine or used for immunohistochemistry. To improve antigen reactivity, sections were immersed in antigen retrieval citrate buffer (Dakocytomation, Glostrup, Denmark) and incubated at 97 °C for 45 min in a water bath. Slides at RT were washed three times in 0.05 % Tween/PBS for 5 min and then in PBS. Endogenous peroxidase was neutralized in 1.5 *v/v*% H_2O_2 in d.w. during 15 min. Slides were washed in d.w., rinsed 3-times in 0.05 %Tween/PBS and left in PBS. Non-specific binding was blocked in 5 % normal goat serum in PBS for 10 min. Tissue sections were washed in 0.05 %Tween/PBS as previously and incubated with primary antibodies for 30 min at RT: mouse anti-P16 (Medical Biocare INK4a) was used undiluted, and mouse anti-Bcl-2 (Dakocytomation, clone 124) at 1/100 dilution in primary antibody diluting buffer (Biomed Corp. Forster city, CA). Sections were rinsed in 0.05 % Tween/PBS) then were incubated in post primary block solution for 30 min (Novolink TM max polymer detection system from Novocastra laboratories, ltd Balliol Business Park west, Benton Lane, Newcastle Upon Tyne NE 12 8EW UK). Following washing in 0.05 % Tween/PBS and PBS they were incubated in Novolink™ polymer for 30 min and rinsed as previously. Peroxidase activity with revealed with DAB (Diaminobenzidine) substrate working solution (50 μl DAB chromogen in 1 ml of Novolink™ DAB substrate buffer) for 10 min. The preparations were rinsed in PBS and then in d.w. and the sections were counterstained with Harris hematoxyline solution for five min. Negative controls were prepared by omitting the primary antibody from the reaction mixture. The slides were mounted in Eukitte and analysed under a Leitz microscope equipped with a CCD camera and an image analyser (Vysis software).

Results

Normal Placenta Development

Pictures were taken of the best tissues consciously selected for each antibody and each pathologies, rather than the same specimen of placenta moles and choriocarcinomas. The staining specificity does not change between the different specimen of the same pathology or structure. The changes were associated with the more or less good preservation of

the tissue. With this presentation option, the staining comparisons were easier to examine.

p16 staining was localized in the nucleus of the CTB and the extravillous placenta starting around 8WGS and in the villous tissues in the nucleus of mesenchymal cells (Fig. 1, 1). The number of positive nuclei increased during development and reached a maximum around 17WGS (Fig. 1, 2–3). During this period a weak staining appeared in the STB cytoplasm, too. In the extravillous placenta this staining started to appear at the base plates of placental columns (proliferation zone), i.e. in proximity of CTB, and appeared in a scattered pattern in the distal (invasive) zone (Fig. 1, 1). P16 expression returned to a low level again at full term (Fig. 1, 4), but differentiation of the CTB at this period made nuclear counting difficult due to the reduced CTB mass. In the extravillous cells of the placenta, the number of positive nuclei was rare. In the mesenchyme endothelial staining was identified in a few vessels irrespectively of their size (Table 2).

Concerning the decidua, we had access only to the superficial zone. Here, CTB cells, penetrating the caduc (interstitial trophoblast) showed cytoplasmic staining. CTB cells colonizing the uterine spiral arteries were sparse but also showed positive cytoplasmic staining (Table 2). These staining patterns were stable during 8–17 WGS. However, the p16 reactivity dramatically declined in interstitial trophoblasts around the full term of gestation (at 39 WGS) when the quasi-totality of cells became negative. The staining of CTB cells in the spiral artery wall was weak or absent.

Bcl-2 staining was always present in the cytoplasm of STBs irrespectively of the developmental stages analysed. This reactivity increased during trophoblast differentiation and extended to the CTB during 17–39 WGS (Fig. 2, 2–4), whereas the staining was strictly located in the STB at 8 WGS (Fig. 2, 1). In the extravillous placenta, Bcl-2 was detected exclusively in STB (Table 2).

In the decidua (Table 2), interstitial CTB cells and the uterine artery wall were weakly positive through 8–17 WGS. This staining was more frequent around week 17. However, the uterine glands were always positives at these stages. Towards 39 WGS CTB cells which invaded the superficial part of the decidua were either negative or slightly positive and only few cells from the uterine glands were positives. Therefore, at this stage, Bcl-2 labelling became very weak or absent.

Hydatidiform Moles

Three of these moles presented a recurrent and familial transmission (M251; M264 1 and 2). The cytogenetic analyses showed that these samples were diploid and had a mutation in the NLRP7 gene (Table 1 and R. Slim). These moles corresponded to partial hydatidiform moles (2nPHM).

p16 reactivity was present in the CTB cell nuclei with variable frequencies, depending on the hydatidiform mole phenotype, but the staining was never strong and was frequently erratic (Fig. 1, 5). The staining was homogenous in proliferating cytotrophoblast cells at the extravillous trophoblast and the frequency of positive nuclei was high. We could distinguish a higher staining in the proximal parts compared to the distal region within these extravillous structures (Table 2 and Fig. 1; 6–8). The frequency of stained nuclei was maximum in the invHM where some cells in the villous stroma were also positive (Table 2 and Fig. 1; 9–11). In the case of 2nPHM, we observed a mild cytoplasmic STB staining (Fig. 1; 7). In the case of the two twin pregnancies from weeks 15–16 (M246, M247 and M253, M254), the CHM and the normal placenta showed the same labelling pattern similarly to the control age-matched placenta.

Bcl-2 reactivity was identified in the STB cell cytoplasm from PHM, it was homogenous and corresponded to the staining pattern found in the normal mid-gestation placenta (Table 2 and Fig. 2; 5–6). In CHM, this STB staining presented some variations associated with villous histological state. The CHM villi were modified by the molar pathology and expressed less Bcl-2, (Fig. 2; 7–8). In the twin pregnancies we could not found differences between the normal and affected trophoblastic tissue. In invHM, the STB cytoplasm from the metastatic villous fragments which invaded the lungs, Bcl-2 was strongly expressed (Table 2 and Fig. 2; 10). In the case where this invasion was limited to the myometrium this expression was more partial (Fig. 2; 9).

We have not obtained caduc tissues from CHM, therefore our results concern only caduc tissues from other types of moles (Table 1). Few p16 was expressed in these maternal tissues. Nuclear staining was evident in some CTB cells which invaded the caduc, except in invHM. In the PHM a nuclear labelling in the uterine glands cells was observed (Table 2). Bcl2 appeared weakly and variably in some CTB cells found in the decidua and in the uterine gland epithelium. This last staining was more common in PHM.

Choriocarcinomas

None abnormalities, gain or loss, were observed at p16 9p21 and Bcl-2 18q21.3 chromosome regions by array CGH high resolution 244K.

p16 nuclear reactivity was found in the mesenchyme of disorganized villosities. Some CTB cells expressed a non-common nuclear staining associated or not with cytoplasmic labelling. This staining corresponds to the pattern observed in the early (8WGS) normal trophoblasts, (Table 2 and Fig. 1; 12–15).

Bcl-2 reactivity was weak and found only in the STB cytoplasm which was similar to the staining in the normal embryonic trophoblast. In the trophoblastic proliferation the

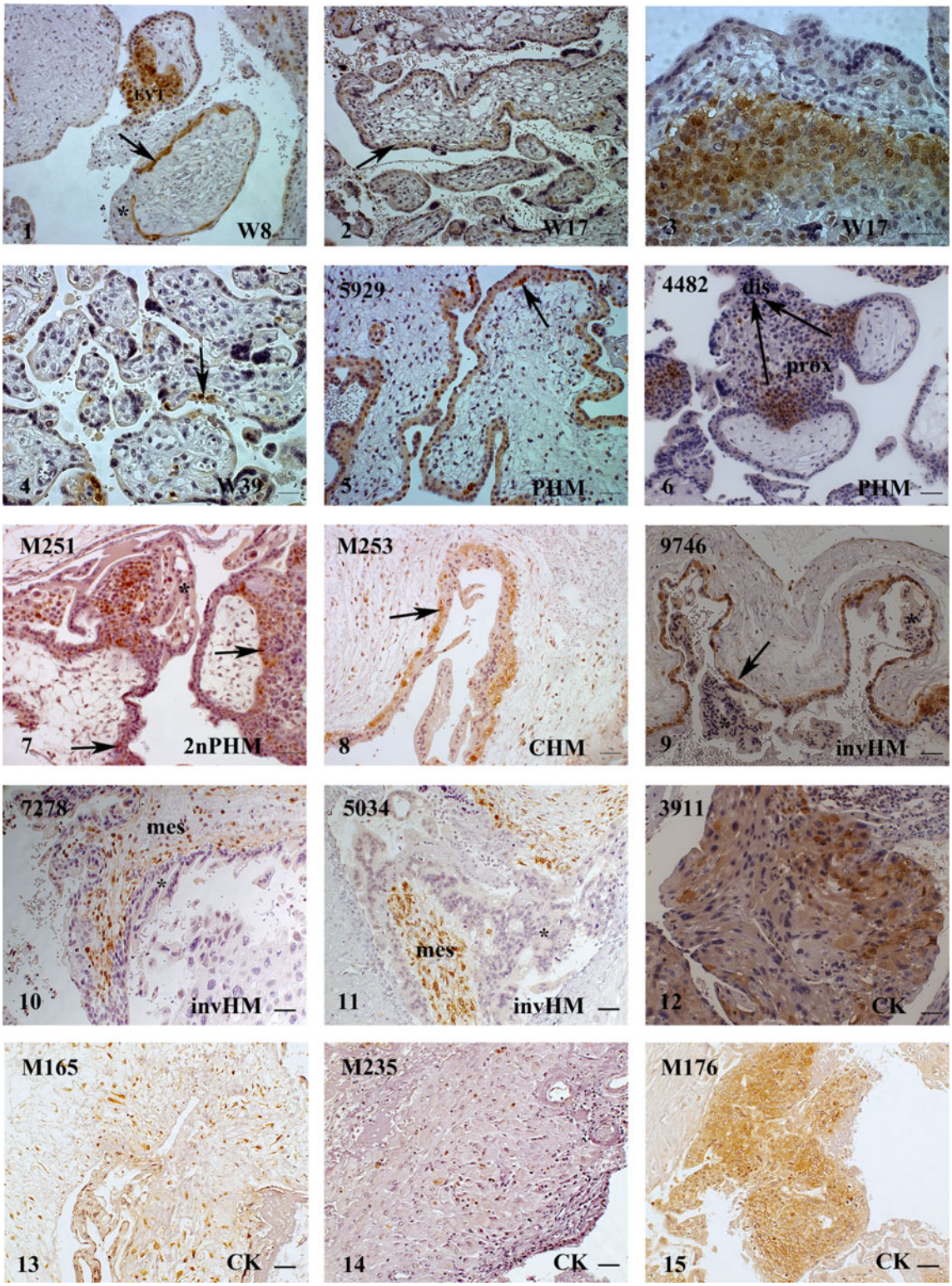

Fig. 1 p16 staining during normal trophoblast development and in molar pathologies (PHM, CHM, invHM, CK). During normal trophoblast differentiation from w8 to w17, CTB nucleus and villous mesenchyme were stained (1–3), at w39 the labelling was slight, only few nucleus were stained (4). Single arrow: CTB; Star: STB. In the PHM and CHM we can observe CTB nucleus staining (villous and extravillous) (5–8). A weak staining can also be observed on STB of 2nPHM (*, 7). This nucleus labelling is more frequent in the proximal part of the EVT (prox) than in its distal part (dis) (6). In the invHM these are the invasive villous fragments which invade the myometer (9) or the lung (10–11) which are stained in the CTB and in the villous mesenchyme (mes). In all cases of CK, mesenchyme (12, 13, 14) and the proliferations of CTB cells (15) were stained. The CK 12 is an infiltrating choriocarcinoma. Mes: villous mesenchyme. Bars=50 μm

Table 2 (1,2,3): Expression of p16 and Bcl-2 genes during the human placenta development, hydatidiform moles, choriocarcinoma and decidua. Without indications p16 staining is nuclear type

1. antibodies	p16					Bcl-2				
placenta	CTB		STB	Mes		CTB		STB	Mes	
	villous	E-Vil		C	ve	villous	E-Vil		C	ve
W 8/10	+	+	−	+	−	−	−	+	−	−
W 15/18	++	++	+/−cyto	+	−	−	−	++	−	−
W 37/39	+/−	+	−	…	+/−	−	−	+++	−	−
2. antibodies	p16					Bcl-2				
moles/chorio	CTB		STB	Mes		CTB		STB	Mes	
	Villous	E-Vil				Villous	E-Vil		C	ve
PHM tripl	+	+	−	−	−	−	−	++	−	−
PHM 2n	+	++	+cyto	−	−	−	−	++	−	−
CHM	+	+	−	+/−	−	−	−	+/++	−	−
inv HM	+/− to ++	+ to ++	−	+/++	−	−	−	+++	−	−
Chorio	+/− to +	+/− to +	+/−cyto	+	−	− (cells)	− (cells)	+	−	−
3. antibodies	p16					Bcl-2				
decidua	CTB	Spiral art	gland	end		CTB cells	art	gl	end	
W 8/10	+	+/−	−	−		…/+	…	+	−	
W 15/18	+	+/−	−	−		…/+	…/+	+	−	
W 37/39	−	+/−	−	−		−	…	+/−	−	
PHM tripl	+/−	−	…	−		…	−	++	−	
PHM 2n	+/−	−	+	−		+/−	−	+	−	
inv HM	−	−	…	−		+/−	−	+	−	
Chorio	−	−	−	−		−	−	+/−	+	

Cyto Cytoplasmic staining, *Mes* Mesenchyme, *C* Villous mesenchyme cells, *ve* Vascular endothelium, *E-vil* Extra-villous trophoblaste of the column, *Chorio* Choriocarcinoma. …, +/−,+,++,+++: number of staining cells in the corresponding tissue, rare; <10 %; 20/30 %; 50 %; >80 %. end: uterine vessels other than spiral arteries (spiral art). The staining of spiral arteries corresponds to trophoblast cells migration in the wall of these vessels

specific cytoplasmic staining was present (Table 2 and Fig. 2; 11–12).

Discussion

Morphogenesis and Remodelling of the Pathologic Placenta

Remodelling of the villous placenta at the end of first trimester is of vital importance for the increasing, harmonious supply of expanding embryo/foetus with oxygen and nutrients. Here we document that during this remodelling ("villous reshaping") both p16 and Bcl-2 were expressed at low level allowing tissue expansion and simultaneous apoptotic cell death to progress in parallel. Our results are supported by recent findings obtained in human [37] suggesting that remodelling of villous placenta during the first trimester requires a complex homeodynamics involving CTB associated with syncytialization and apoptosis in STB. Around mid-gestation (17 WGS) the p16 protein turned to be highly expressed in the nuclei of normal CTB and EVT cells. The p16 expression kinetics correspond to villus reshaping and it was associated with apoptosis of luminal epithelial cells and decidua, coordinating decidualization of the endometrium and invasion of trophoblastic cells in mouse [6]. This proliferation control could be linked to p16 functioning which veto cell cycle progression at the G1 phase via specific binding to Cd4 (cyclin dependent kinase 4) and inhibition of the catalytic activity of cyclin-D complex [1, 38] allowing the full terminal differentiation to occur in the STB.

In normal placenta, when differentiating towards an invasive phenotype, EVTs stop proliferating (high p16 expression), loose intercellular adhesion molecules (E-cadherin), down-regulate several junctional proteins (connexins and integrins) and express distinct surface determinants like HLA-G and specific hormones receptors, hPL (phenotype switch) [39]. So, in healthy conceptus the role of p16 protein is to veto the villous proliferation thus facilitating the invasion process.

In molar pathology, p16 was expressed in CTB cells in the case of invHM and in the EVT in the cases of 2nPHM and invHM. Therefore, these expression patterns phenocopie what was observed in the normal trophoblast. In these

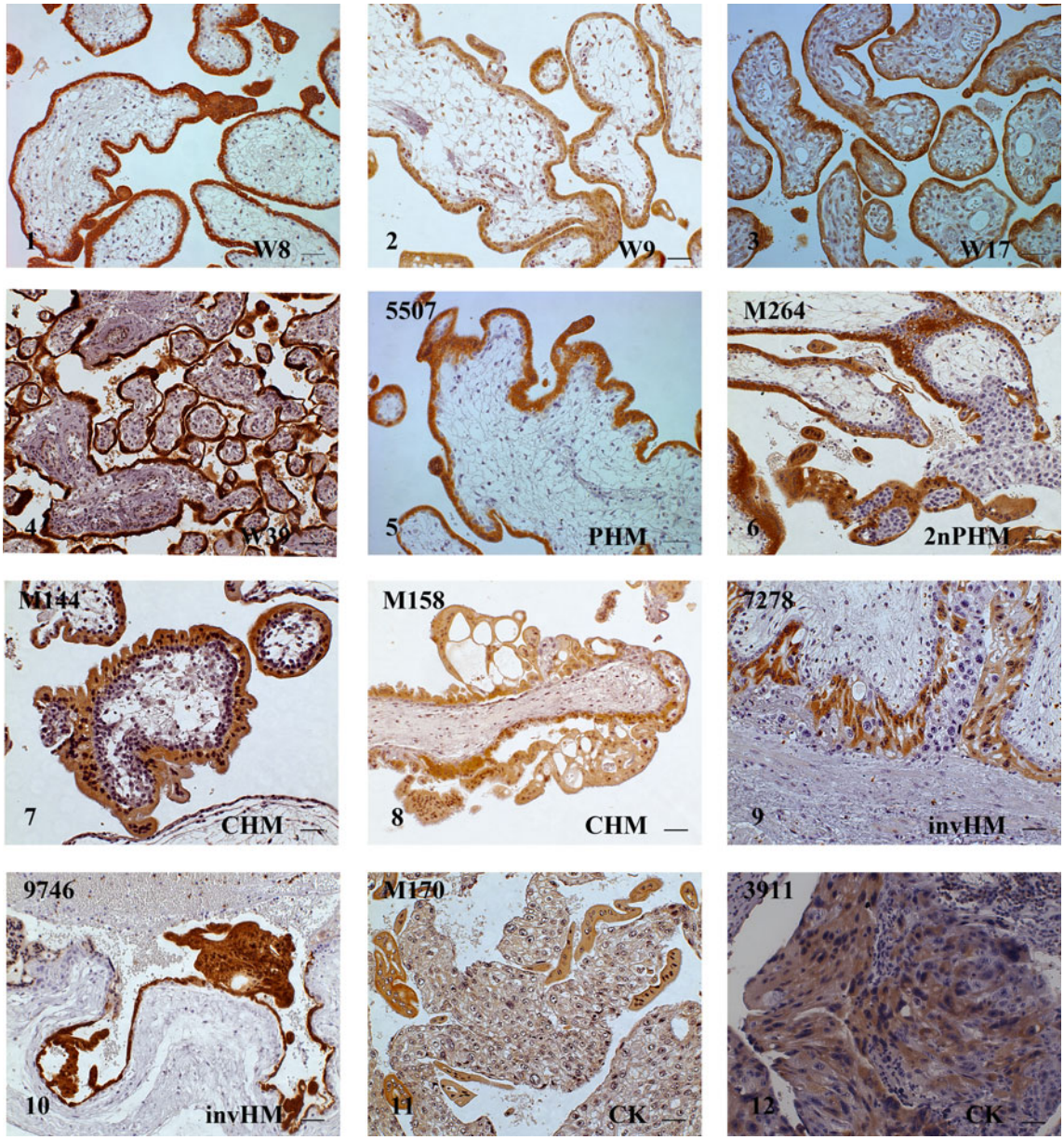

Fig. 2 Bcl-2 staining during normal trophoblast differentiation from w8 to w39 and in different molar pathologies. In all cases only STB cytoplasm was stained. During the normal trophoblast differentiation, this cytoplasm staining increases gradually from w8 to w39 (1–4). We can observe the same staining in the PHM (5–6). In the CHM (7–8) the STB cytoplasm staining was often less intense. In the invHM the STB labelling presents a great development as well when the villous fragments were invaded the myometer (9) or the lung (10). The CK (11–12) were stained only in the STB cytoplasm. (12): infiltrating CK. This last staining can be compared to that of Fig. 1 image 12. Bars=50 μm

proliferative locations the CTB cells become invasive but gradually lose their proliferative function allowing differentiated villi to integrate into the uterus wall from the beginning of gestation. The pathologic situation could be distinguished by the p16 reactivity in the villous stroma which was weak in the normal stroma. In addition, the number of positive nuclei increased gradually through progression of CHM to invHM. But, not all invHM expressed the p16 protein. We speculate that the frequency of p16 expression in the villous stroma would be the sign of progression toward an invasive phenotype in the case of invHM. Meanwhile p16 expression, alone, cannot explain the invasive phenomenon since we never observed such cell migrations in PHM.

Interestingly, the STB cytoplasm was negative in the molar villi at 17WGS in contrast to controls and 2nPHM. This explain why cell fusion, providing STB integrity was merely preserved in these moles while 2nPHM were similar to controls. In addition, CTB functioning was different in 2nPHM and the other moles because only these 2nPHMs

resulted from a NLRP7 gene mutation and had a biparental karyotype.

Remarkably, the mechanism of invasion is different in invHM as compared to the control placenta. In controls, the migration of CTB cells depends on the villus-embedding into the receptive endometrium, in contrast to invHM where neoformed villus fragments invade further on the uterine arteries.

This work reveals that during the molar process the kinetic pattern of p16 expression corresponds to a precise regionalization, rather than to a decreased expression. Although Xue at al. [10] reported hypermethylation of the p16 gene promoter during molar pathogenesis, resulting gene silencing, our studies show instead re-allocation of p16 specific areas.

The Syncytiotrophoblast

In normal STB the weak Bcl-2 expression during the first trimester indicates for ongoing apoptotic cell death and in turn the subsequent rapid increase in Bcl-2 reactivity reflects for a gain of anti-apoptotic behaviour in the trophoblast layer [37]. This functional switch occur simultaneously with cell fusions required for harmonious development, as shown elsewhere [4, 40, 41]. The robust Bcl-2 expression in normal STB at the end of pregnancy was in contrast with some publication [18]. The authors consider that lack of Bcl-2 function in full term placenta could favour delivery. Our results indicated that Bcl-2 down-regulation during the end of pregnancy must be abrupt and non progressive, since at 39WGS we could not observe such a decrease, similarly to Kim and co-workers [12]. In the moles, Bcl-2 staining phenocopies the mid-gestation placenta (17WGS). STB was always well differentiated, including the invHM. Thus, the cell fusion process was not impaired at the beginning of these pathologies. Yet, CHM was characterized by villi with reduced vasculature, resulting hypoxic tissue environment [42]. Tissue hypoxia induces apoptosis in trophoblastic cells via Bcl-2 suppression [43, 44]. In our study the decrease of Bcl-2 in CHM was not generalized which could be explained, at least in part, the presence of blood vessels in these moles. The highest expression of Bcl-2 in invHM with regards to other moles, may be involved in maintaining the integrity of molar villi during their migration towards other anatomical allocations (myometrium, lung) allowing their integrity and development. Bcl-2 expression in caduc uterine glands of normal or molar placenta is compatible with antigenic similitude between uterine glands and STB, and with their persistent involvement in the good progression of pregnancy. On the other hand, the weak Bcl-2 reactivity in molar CTB cells which were migrated to the superficial decidua could indicate for low survival rate. Importantly, the villous fragments (high Bcl-2 reactivity) in one hand, which invade the arteries, and the trophoblastic cells (weak Bcl-2 reactivity) on the other hand, which invade the endometrium, are distinct pathophysiological entities.

Choriocarcinoma

Expression variations of p16 and Bcl-2 genes observed in the choriocarcinoma did not come from chromosomes gain or loss acquired during the cancer process. In fact none modifications on the level of the loci of these genes was observed in the tested cases with array CGH [45].

Bcl-2 expression which was specific for STB, highly decreased in the choriocarcinoma as compared to the moles (PHM; CHM ; invHM). Previous reports indicated for a possible role of apoptosis in the regulation of placental growth and function and the role of anti-apoptotic Bcl-2 protein family in controlling cell survival and death [14]. Expression of Bcl-2 protein in EVT along the invasion of the decidua may be one of the major factors preventing these structures from early cell death, which is indispensable for the maintenance of pregnancy. Inversely, the increased frequency of apoptosis in invasive EVT may be attributed to decreased Bcl-2 expression in term placenta [14, 18, 46, 47]. The lack of Bcl-2 protein in choriocarcinoma STB could also explain the disorganization of the villous structures and the prevailed development of proliferation sites (weak p16 staining). These results consolidate observations [48] which showed that in choriocarcinoma the increase of apoptosis occur simultaneously with increased proliferation.

In summary, the trophoblastic migrations in invHM and choriocarcinoma, seems to be associated with the antagonistic properties of p16 and Bcl-2 proteins, respectively. When villous fragments were protected from apoptosis (Bcl-2 positive), and show an invasive potential (p16 high), the trophoblast disorganization would be directed towards an invHM. When STB is less,-or non-protected (Bcl-2 weak), and cell proliferation is high (p16 weak), the trophoblast disorganization would be directed towards a choriocarcinoma. The severity of choriocarcinoma goes hand in hand with high cell turnover, which would favour the invasive capacity of cells; this is distinct in the case of invHM. However, this particularly representative sample of the different moles did not allow a reliable statistical study. Hence, it would be of interest to obtain more samples in order to quantify the various results and to corroborate our vision on the hydatidiform moles and post molar choriocarcinomas invasiveness.

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