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Immunoexpression of the hepatocyte growth factor (HGF), HGF-receptor (c-met) and STAT3 on placental tissues from malformed fetuses

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Summary. To characterize the possible relationship between the expression of the HGF/HGF-R system with transcription factor STAT3, responsible for morphogenetic response of HGF stimulation, and the embryonic development alterations, we investigated, by immunohistochemistry, the expression of HGF, c-met and STAT3 in 9 placentas from malformed fetuses and 9 control placentas from non-malformed fetuses.

The major and distinct patterns of expression characterizing the placentas from malformed fetuses were a higher percentage mean of stromal cells stained for HGF, c-met and STAT3 antibodies (60%, 66% and 54%, respectively on fibroblast cells and 44%, 57% and 42%, respectively on myofibroblast cells) and a lower percentage mean of cytotrophoblast cells stained for the same antibodies (2%, 2% and 1%, respectively), than in control placentas. In fact, in this latter group, the stromal fibroblast cells were stained in a percentage mean of 27%, 22% and 7%, respectively; the stromal myofibroblast cells in a percentage mean of 5%, 6% and 2%, respectively and the cytotrophoblast cells in a percentage mean of 25%, 34% and 18%, respectively.

The expression of each antibody on stromal cells in both groups suggests an alternative role of the HGF/HGF-R system activating the via STAT3 transdution and operating on placental tissues, overall in organogenesis alteration conditions.

This immunohistochemical approach could be used in the diagnostic practice of pathologists on chorionic villi biopsy when genetic alterations are absent and ultrasound aspects are doubtful for malformations.

Key words: HGF, c-met, STAT, Placenta, Immunohistochemistry

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Introduction

Hepatocyte growth factor (HGF), a pleiotropic mesenchyme-derived cytokine, originally considered as a mitogen for hepatocytes *in vitro*, is a potent stimulator of both proliferation and DNA synthesis in receptive cells, including trophoblast (Michalopoulos et al., 1984; Tajima et al., 1992; Saito et al., 1995). Moreover, HGF, also known as scatter factor, is able to induce morphogenesis and epithelial differentiation during embryogenesis (Weidner et al., 1991; Bhargava et al., 1993; Brinkmann et al., 1995). HGF exerts its biological effects via binding to c-met, a protooncogene which is a member of the trans-membrane growth factor receptor tyrosine kinase family (Bottaro et al., 1991; Bardelli et al., 1994; Ponzetto et al., 1994). The consequent molecular mechanisms to the interaction between HGF and c-met, have not been completely clarified. However, it is known that the scattering response to HGF is dependent on PI3-kinase and Ras-Rac/Rho pathways activation; the growth response is related to the Ras-mitogen-activated proteins and morphogenetic effects are mediated by the “signal transducers and activators of transcription” (STAT), a family of transcription factors that bind specific DNA elements (Ridley et al., 1995; Royal and Park, 1995; Ponzetto et al., 1996; Boccaccio et al., 1998; Decker and Kovarik, 1999). Both HGF and c-met genes are essential for the placental development in the second part of gestation in mice, as well as for the complete morphogenesis of epithelial tissues and the formation of organs such as lung, kidney and brain (Birchmeier et al., 1993; Jung et al., 1994; Uehara et al., 1995; Kolatsi-Joannou et al., 1997; Sakurai et al., 1997; Ohmichi et al., 1998). During fetal life, the placenta synthesizes several growth factors, including HGF, and indeed placental villous core is an important source of both fetal and maternal plasma HGF levels (Wolf et al., 1991; Wang et al., 1994; Khan et al., 1996; Furugori et al., 1997; Kauma et al., 1997; Kolatsi-Joannou et al., 1997).

In the present report we investigate the immunohistochemical expression of HGF, c-met and STAT3 on placentas taken from pregnancies of malformed fetuses to study a possible relationship between the placental expression of HGF/c-met system with its activated transcription factor and fetal morphogenetic alterations.

Materials and methods

Tissue collection

Nine placentas from pregnancies (ranging from 19-24 weeks of gestation) which resulted in fetuses with malformations regarding ectoderm, mesoderm and endoderm line alterations, were selected from files of the Department of Human Pathology, University of Messina, Italy (Table 1). A second group of nine placentas from voluntary abortions of physiological pregnancy (ranging from 19-24 weeks of gestation) which resulted in non-malformed fetuses were also taken as controls (Table 1). In all placentas the appearance of chorioamnionitis was excluded on the bases of macroscopic and histological observations. Full-thickness placental samples were removed from an off-center position, halfway between the umbilical cord and the edge of the placentas. Tissues were fixed in 4% formalin before embedding in paraffin wax by standard techniques. Haematoxylin-eosin- and periodic acid-Schiff (PAS)-stained sections of each specimens were performed.

Pathological evaluation

The histological maturation of chorionic villi was determined by assessing the age-maturation villi (stem, mesenchymal, immature and mature intermediate and terminal villi). Calcifications and fibrinoid necrosis of the chorionic villi were assessed as absent, focal or widespread. Vascularization was evaluated as normal, increased or decreased. Basement membrane was evaluated in PAS-stained sections as normal or thickened (focal or widespread thickening).

Immunohistochemistry

Serial five-micrometer sections of the selected blocks were deparaffinized in xylene, rehydrated and then the endogenous peroxidase activity was quenched by addition of 3% hydrogen peroxidase for 15 min. Immunohistochemical procedures were performed by polyclonal rabbit antibodies raised against HGFα (H-145, 1:100; Santa Cruz Biotecnology, Inc., Santa Cruz, CA), c-met (p140 anti h-met, 1:100; Santa Cruz Biotecnology, Inc., Santa Cruz, CA), STAT3 (h-190, 1:100; Santa Cruz Biotecnology, Inc., Santa Cruz, CA), by mouse monoclonal antibodies raised against desmin (D33, 1:100; Dako Corporation, Carpinteria, CA) and α-smooth muscle actin (1A4, 1:100; Dako Corporation, Carpinteria, CA), using the biotin-streptavidin-peroxidase method (LSAB kit from Dako Corporation, Carpinteria, CA). The reaction was developed by 3,3'-diaminobenzidine (DAB) with 0.05% hydrogen peroxide as chromogen. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Specificity was assessed by omitting the primary antiserum or by replacing the primary antiserum with normal rabbit or mouse serum or by preincubating the primary antiserum with HGF (Santa Cruz Biotechnology), c-met (Santa Cruz Biotechnology), STAT3 (Santa Cruz Biotechnology), desmin (Dako Corporation, Carpinteria, CA) or α-smooth muscle actin (Dako Corporation, Carpinteria, CA).

For the evaluation and comparison of the results using HGF, c-met and STAT3 antibodies, the following criteria were used: (1) number of positive cases; (2) number of positive syncitiotrophoblast, cytotrophoblast, stromal fibroblast and myofibroblast cells per case; the count of the number of reactive cells was based on evaluation of 1000 cells for each case, using a x50 magnification; (3) site of positivity: cytoplasmic, and/or membranous, and/or nuclear only for cytotrophoblast and syncitiotrophoblast cells; and (4) semiquantitative grading of staining, using score system from 0 to 4 (0=

Table 1. Placental samples obtained from pregnancies of malformed fetuses and control placentas.

No. OF CASES OF PLACENTAS FROM MALFORMED FETUSES	AGE (weeks)	MALFORMATION TYPE	LINE ALTERATION	No. OF CASES OF CONTROL PLACENTAS FROM NON-MALFORMED FETUSES	AGE (weeks)
1	21	Agenesis of the corpus callosum		1	21
2	22	Dandy-Walker syndrome	Ectoderm	2	22
3	24	Dandy-Walker syndrome		3	24
4	19	Renal bilateral agenesis		4	19
5	21	Renal dysplasia	Mesoderm	5	21
6	23	Renal bilateral agenesis		6	23
7	22	Extralobar sequestration		7	22
8	22	Pulmonary total agenesis	Endoderm	8	22
9	24	Pulmonary unilateral agenesis		9	24

absent; 1+ = weak; 2+ = moderate; 3+ = intense; 4+ = very intense). Immunoreactions for desmin and α-smooth muscle actin antibodies were used to design the placental stromal fibroblast and myofibroblast cells, respectively (Castelluci and Kaufmann, 2000).

Histological, histochemical and immunohistochemical evaluations of all placentas were done twice and blindly by three different pathologists (M.T., M.G., G.B.) with an inter-observer concordance of 100%.

Statistical Analysis

Immunohistochemical results were evaluated using the Mann-Whitney test to compare unpaired groups. All calculations were performed using a computer analysis package (Prism 2.0, GraphPad Software San Diego, CA).

Results

Histology

Comparable features about stem, mesenchymal, immature and mature intermediate and terminal villi were observed in placenta of malformed fetuses as well as in control placenta having the same pregnancy age. Calcifications and fibrinoid necrosis were absent and the angioarchitecture of villi was normal for pregnancy age in all placentas. No thickening of basement membranes was detected in malformed placentas or in control placentas.

Immunohistochemistry

All immunohistochemical results are summarized in Table 2 and in Table 3. All placentas from malformed and non malformed fetuses expressed HGF and c-met and STAT3 at different levels, except for syncitiotrophoblast which did not express STAT3.

In stromal fibroblastic cells from malformed fetus placentas, the expression of HGF, c-met and STAT3 was detected with a percentage mean of 60%, 66% and 54%, respectively (Table 2). The stain grade was: 3+ for HGF and STAT3, and 4+ for c-met (Table 3; Fig 1A, 2A and 3A). In all control placentas, stromal fibroblasts were stained with a lower percentage mean (27% and 22% for HGF and c-met, respectively) that observed in placentas from malformed fetuses (Z=3.604, 3.335, respectively and P=0.000 for both) (Table 2). Stromal fibroblasts showed a grade of reactivity ranging from 3+ to 2+ for HGF as well as for c-met (Table 3). In control placentas, the immunoreactivity for STAT3 in stromal fibroblast cells was observed in a percentage mean of 7% (Z=3.601 and P=0.000 comparing both placenta groups) and the grade stain ranging from 2+ to 1+ while only two cases were unreactive (Tables 2, 3).

In stromal myofibroblast cells of malformed fetus placentas, HGF, c-met and STAT3 stain was detected in a percentage mean of 44%, 57% and 42%, respectively (Table 2). The stain grade for HGF and STAT3 ranged

Table 2. Percentage prevalence of HGF, c-met and STAT3 expression on placentas from malformed fetuses and control placentas.

	MIN	MAX	MEAN	MANN-WHITNEY TEXT		MIN	MAX	MEAN	MANN-WHITNEY TEST	
				Z	P=				Z	P=
F.C.						**M.C.**				
HGF										
Malformed fetus placentas	40%	80%	60%	3.604	0.000	20%	70%	44%	3.557	0.000
Control placentas	20%	30%	27%			0%	10%	5%		
c-met										
Malformed fetus placentas	30%	90%	66%	3.335	0.000	30%	80%	57%	3.580	0.000
Control placentas	10%	40%	22%			0%	10%	6%		
STAT3										
Malformed fetus placentas	20%	80%	54%	3.601	0.000	10%	70%	42%	3.560	0.000
Control placentas	0%	10%	7%			0%	5%	2%		
C.C.						**S.C.**				
HGF										
Malformed fetus placentas	1%	5%	2%	3.606	0.000	1%	1%	1%	3.773	0.000
Control placentas	15%	60%	25%			5%	20%	9%		
c-met										
Malformed fetus placentas	1%	5%	2%	3.604	0.000	1%	1%	1%	3.775	0.000
Control placentas	15%	70%	34%			10%	30%	18%		
STAT3										
Malformed fetus placentas	1%	1%	1%	3.767	0.000	0%	0%	0%	-0.136	0.892
Control placentas	10%	40%	18%			0%	0%	0%		

The number of cases are significant to use Mann-Whitney test. Differences between groups are significant (P=0.000) with the exception of STAT3 expression in syncitiotrophoblast cells. F.C.: Fibroblastic cells; M.C.: Myofibroblastic cells; C.C.: Cytotrophoblastic cells; S.C.: Syncytiotrophoblastic cells.

from 4+ to 3+, while for c-met it was always 4+ (Table 3; Figs. 1A, 2B, 3A). The immunoreactivity of stromal myofibroblast cells in control placentas for HGF, c-met and STAT3 was observed in a percentage mean of 5%, 6% and 2%, respectively; lower than in malformed fetus placentas (Z=3.557, 3.580, 3.560, respectively; P=0.000 for each). The stain grade ranged from 3+ to 2+ for HGF, was 3+ for c-met and ranged from 2+ to 1+ for STAT3. In this control group negative results for HGF, c-met and STAT3 were obtained (Tables 2, 3).

Cytotrophoblast cells in placentas from malformed fetuses expressed HGF, c-met and STAT3 with a percentage mean of 2%, 2% and 1%, respectively (Table 2). The immunostaining for each antibody was located on the membrane and cytoplasm and the stain grade was 1+, for both HGF and c-met, and ranged from 2+ to 1+ for STAT3 (Table 3). In control placentas the cytotrophoblast cells were stained with a percentage mean of 25%, 34% and 18% for HGF, c-met and STAT3, respectively; higher than in malformed fetus placentas (Z=3.606, 3.604, 3.767, respectively; P=0.000 for each) (Table 2). The immunoexpression was located on the membrane and cytoplasm for both HGF and c-met; STAT3 immunostaining was evident on the cytoplasm and, occasionally, on the nucleus (Figs, 1B, 2C, 3B). The stain grade ranged from 4+ to 1+ for HGF, from 4+ to 2+ for c-met and from 3+ to 1+ for STAT3 (Table 3).

The syncytiotrophoblast cells in placentas from malformed fetuses expressed only HGF and c-met stain with a percentage mean of 1%. Both immunoreactions were located on the membrane and cytoplasm with 1+ stain grade. The syncytiotrophoblast cells in control placentas showed HGF and c-met stain with a percentage mean of 9% and 18%, respectively; higher than in placentas from malformed fetuses (Z=3.773, 3.775, respectively; P=0.000 for both). The immunostaining for both antibodies was located on the membrane and cytoplasm with a stain grade ranging from 3+ to 2+ (Table 3; Figs. 1B, 2D). Syncytiotrophoblast cells were unreactive for STAT3 in

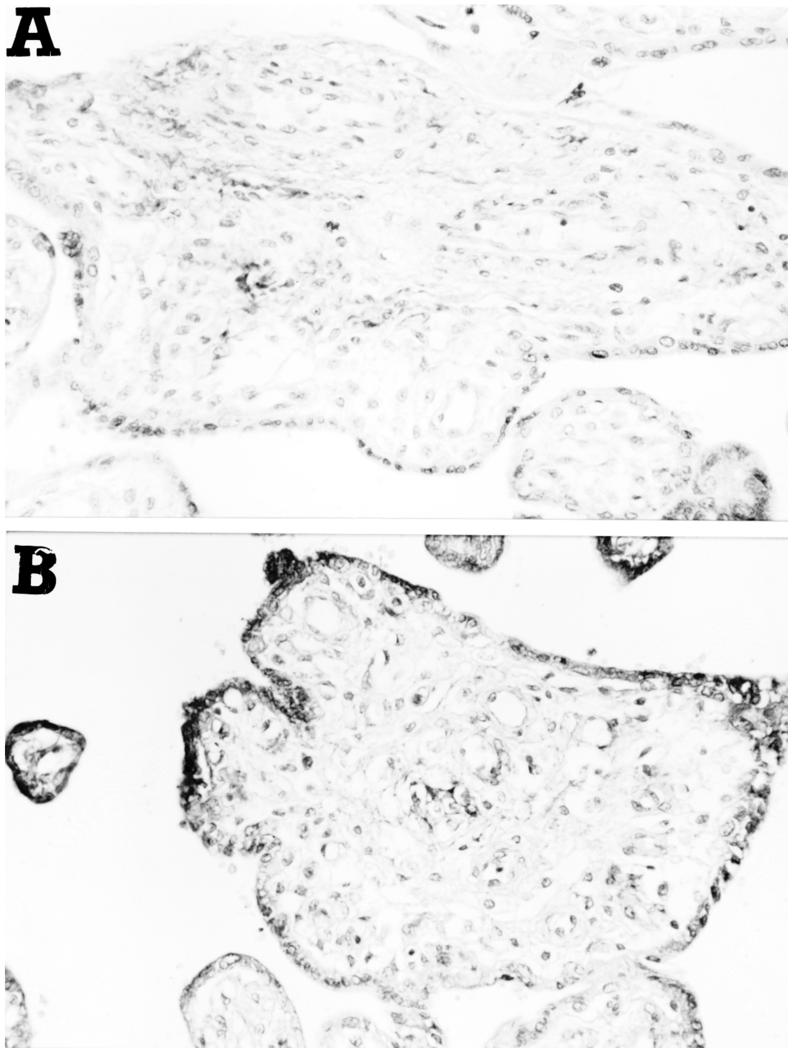

Fig. 1. A. Intense (3+) HGF immunoreaction on fibroblast cells (arrow) as well as on myofibroblast cells (arrowheads) of malformed fetus placenta (case no. 6). Note the absence of HGF stain on cytotrophoblast cells. x 100. **B.** Very intense (4+) HGF immunoreaction on cytotrophoblast cells (arrow) of control placenta (case no.1). x 200

both groups of placentas (Table 2, 3).

Hofbauer cells showed positive immunoreaction for HGF, c-met, and STAT3 with the same percentage mean of 2% in placentas from malformed fetuses as well as in control placentas. HGF and c-met stain were located on the membrane and cytoplasm and STAT3 positivity was observed on the cytoplasm and sometimes on the nucleus. In these cells the stain grade was 3+ for each antibody (data not shown).

Table 3. Pattern of expression of HGF, c-met and STAT3 stain in placentas from malformed fetuses and control placentas.

	POSITIVE CASES	STAINING SCORE				
		0	1	2	3	4
Fibroblast cells						
HGF						
Malformed fetus placentas	9/9				9	
Control placentas	9/9			6	3	
c-met						
Malformed fetus placentas	9/9					9
Control placentas	9/9			7	2	
STAT3						
Malformed fetus placentas	9/9				9	
Control placentas	7/9	2	1	6		
Myofibroblast cells						
HGF						
Malformed fetus placentas	9/9				3	6
Control placentas	6/9	3	1	6		
c-met						
Malformed fetus placentas	9/9					9
Control placentas	6/9	3			6	
STAT3						
Malformed fetus placentas	9/9				7	2
Control placentas	6/9	3	2	4		
Cytotrophoblast cells						
HGF						
Malformed fetus placentas	9/9		9			
Control placentas	9/9		3	4	1	1
c-met						
Malformed fetus placentas	9/9		9			
Control placentas	9/9			7	1	1
STAT3						
Malformed fetus placentas	9/9		7	2		
Control placentas	9/9		3	1	5	
Syncitiotrophoblast cells						
HGF						
Malformed fetus placentas	9/9		9			
Control placentas	9/9			7	2	
c-met						
Malformed fetus placentas	9/9		9			
Control placentas	9/9			7	2	
STAT3						
Malformed fetus placentas	0/9	9				
Control placentas	0/9	9				

The stain was scored on a five-point scale from 0 (absent) to 4+ (very intense), as specified under Materials and Methods.

Discussion

In this study, we have demonstrated the immunoexpression of HGF, c-met and STAT3 on placentas from malformed fetuses with some significant differences in their immunoreactive pattern as compared to placentas from non-malformed fetuses. In particular, on stromal fibroblast and myofibroblast cells of placentas from malformed fetuses, we have detected the α chain of biologically active HGF form and c-met in a higher percentage than that obtained in control placentas. Little c-met signal was previously described on the stromal placental cells (Furugori et al., 1997; Somerset et., 1998). In the present study, the simultaneous expression of HGF and c-met antibodies on stromal placenta cells allows us to hypothesize that an autocrine mechanism operating through HGF, among stromal cells, as well as an autocrine and/or paracrine role for HGF and c-met in the mesenchymal-epithelial transition, is present. However, a similar mechanism has been previously suggested in mesenchymal cells during embryonic development in which the co-expression of the *HGF* and *met* genes has been demonstrated; in addition, c-met expression has been observed in immortalized cells from metanephrogenic mesenchyme, without undergoing epithelial conversion (Karp et al., 1994; Woolf et al., 1995).

We found an immunocytochemical signal for HGF on cytotrophoblast and syncytiotrophoblast cells with a prevalent expression in placentas from non-malformed fetuses rather than in malformed fetus placentas. In fact, these placentas the percentage mean of HGF-stained cytotrophoblast and syncytiotrophoblast cells was lower than that observed in the same stained cells in control placentas. HGF immunostaining was located on the membrane and cytoplasm of cytotrophoblast and syncytiotrophoblast cells. Interestingly, we detected HGF-R immunostaining on the membrane and cytoplasm of cytotrophoblast and syncytiotrophoblast cells. These features suggest a possible interaction on the cytotrophoblast and syncytiotrophoblast membrane between ligand (HGF), produced by stroma, and its receptor (c-met), expressed on the membrane of trophoblast cells. With the exception of one report demonstrating HGF mRNA expression on syncytiotrophoblast cells, HGF was revealed only by the immunohistochemistry was revealed on the trophoblast bilayer of the villous while HGF mRNA research was negative (Wolf et al., 1991; Wang et al., 1994; Kilby et al., 1996; Kolatsi-Joannou et al., 1997; Somerset et al., 1998). These data support the hypothesis of HGF/c-met interaction to explain the HGF immunostaining on cytotrophoblast and syncytiotrophoblast membrane and in this way the HGF and c-met stain observed on the cytoplasm of the same cells could be related to conformational changes of two proteins following the formation of HGF/HGF-R complex.

Cytokines and growth factors regulate multiple aspects of development, cell growth and differentiation

through interaction with specific receptors. HGF, binding c-met, leads to dimerization, autophosphorylation and transient association of the receptor with cytoplasmic STAT3 protein (through its SH2 domain). This association induces STAT3 activation by phosphorylation on tyrosine followed by translation to the nucleus (Boccaccio et al., 1998).

STAT3 activation was detected in previous reports in human hepatocytes and in human skin fibroblast cells (Lin et al., 1999; Runge et al., 1999). In human hepatocytes, immunoprecipitation of STAT3 antibody on both cytosolic fraction and nuclear extracts was observed; however, exclusive signal on the cytoplasm was detected, speculating thus a negative modulation of STAT3 activity (Runge et al., 1999). In the present study, we have found co-expression of HGF, c-met and phosphorylated STAT3 overall on cytotrophoblast cells in all placentas from non-malformed fetuses. Our data, confirming previous reports about the location of STAT3 proteins (Lin et al., 1999; Runge et al., 1999), suggest a co-ordinate role of each element of the HGF/HGF-R system with STAT3 in morphogenesis of cytotrophoblast vs syncytiotrophoblast cells in pregnancy of normal fetuses. Therefore, the role of STAT3 may be limited to cytotrophoblast cells because in syncytiotrophoblast cells we did not detect any signal for this antibody. Also, the cytotrophoblast cells of placentas from malformed fetuses expressed STAT3 stain but in a lower percentage mean than in control placentas, without the nuclear location of reactivity. The absence of STAT3 stain on nuclei of cytotrophoblast cells of malformed fetus placentas suggests inadequate activation of transcription factor and/or a negative modulation of STAT3 activity. In placentas from malformed fetuses we detected STAT3

Fig. 2. A) Very intense (4+) c-met immunoreaction on fibroblast cells (reddish color) of malformed fetus placenta (case no. 2). Note the absence of c-met stain on cytotrophoblast cells. x 400. **B.** Very intense (4+) c-met immunoreaction on myofibroblast cells (arrow) of malformed fetus placenta (case no. 7). Note the absence of c-met stain on cytotrophoblast cells and syncytial knots. x 100. **C.** Intense c-met immunoreaction on cytotrophoblast cells (reddish color) of control placenta (case no. 6). Note the absence of c-met stain in stromal cells. x 200. **D.** Intense c-met immunoreaction on cytotrophoblast (reddish color) and syncytiotrophoblast (reddish color and arrow) cells of control placenta (case no. 1). x 200

stain on stromal fibroblast and myofibroblast cells in a higher percentage mean than documented in control placentas. This latter observation may indicate a preferential activation of HGF-c-met-STAT3 pathway on placentas from malformed fetuses.

Comparing the immunostaining location of HGF, c-met and STAT3 in placentas from malformed fetuses with control placentas, interesting differences emerged. In malformed fetus placentas we observed the higher percentage media of stromal cells stained for HGF, c-met and STAT3 antibodies and the lower percentage media of cytotrophoblast cells stained for the same antibodies as detected in control placentas. Moreover, considering the expression of each antibody of the HGF/HGF-R system, in malformed fetus placentas, we detected the c-met stain with a prevalence of cellular expression higher than the HGF stain on stromal cells. Instead, in control placentas we detected the HGF stain more prevalently than the c-met stain only on stromal fibroblast cells. In addition, the higher expression of STAT3 on stromal cells as well as the lower STAT3 stain on cytotrophoblast cells (without stained nuclei) in placentas from malformed fetuses than in control placentas was a peculiar finding of our study. Therefore, a possible role of HGF-c-met-STAT3 placental genes products on organogenesis during fetal life may be hypothesized.

In conclusion, we believe that immunohistochemistry for HGF, c-met and STAT3 can be proposed as an additional useful tool to identify placentas from malformed fetuses. In particular, this immunohistochemical approach can also be used on the placenta of fetal autopsies when post mortem phenomena do not allow the recognition of alterations of the fetal tissues. In addition, the practice of examining chorionic villi by molecular biology investigation can be improved in the negative cases for molecular alterations but is doubtful on the ultrasound basis of fetal malformations.

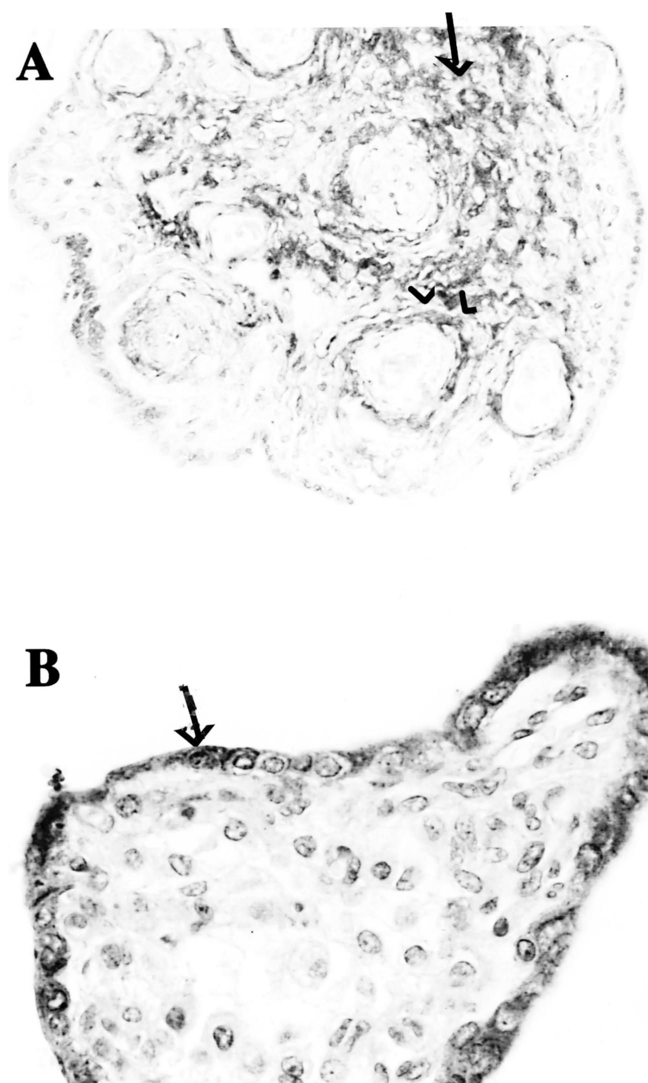

Fig. 3. A. Intense (3+) STAT3 immunoreaction on fibroblast cells (arrow) as well as on myofibroblast cells (arrowheads) of malformed fetus placenta (case no. 3). Note the absence of STAT3 stain on cytotrophoblast cells. x 200. **B.** Intense (3+) STAT3 immunoreaction on the cytoplasm (reddish color) and nucleus (arrow) of cytotrophoblast cells of control placenta (case no. 8). x 400

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