

Short communication

Expression of insulin-like growth factor-II mRNA-binding protein 3 (IMP3) in various normal and neoplastic human tissues

Expresia proteinei tip 3 de legare a ARNm al factorului de creștere similar insulinei II în diverse țesuturi tumorale și non-tumorale

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Abstract

The aim of our study was to evaluate the expression of IMP3 in various normal human tissues, including embryonic tissues and placenta, and also in various human malignancies after testing the optimal immunostaining protocol for mouse monoclonal IMP3 antibody (DAKO, clone 69.1). Material and methods: Representative paraffin blocks, including both tumoral and normal tissues (ovarian, testicular, colon, pancreas, thyroid, skin, lung and adrenal tissue) were selected for immunohistochemistry. Embryonic tissues (a 12th and a 7th week old embryo) and placenta were also evaluated. Immunohistochemistry was performed according to the standard procedures, after a prior standardization of the immunohistochemical protocol for IMP3 using normal human ovary, with known positivity for IMP3. Results: We found that IMP3 is overexpressed in many somatic embryonic tissues, placenta and in some malignant tumors. By contrast, IMP3 level was undetectable in normal human tissues, except in the oocytes of the ovary. Strong and diffuse expression of IMP3 was also demonstrated in anaplastic thyroid carcinoma, while well-differentiated thyroid tumors stained negative for this marker. Conclusion: As IMP3 positive staining was restricted to embryonic and tumor tissue, compared to normal tissue, this molecule may represent a very promising marker for prognostic evaluation in various tumors. Our findings regarding thyroid carcinoma suggest that IMP3 could also be used as a prognostic marker in thyroid carcinoma. However, this is only a hypothesis and further studies on larger number of cases, with long-term follow-up are needed.

Keywords: IMP3, immunohistochemistry, embryo, ovary, anaplastic thyroid carcinoma

Rezumat

Scopul studiului nostru a fost de a evalua expresia IMP3-ului în diverse țesuturi non-tumorale, inclusiv țesuturi embrionare și placentă, precum și în tumori maligne, după punerea la punct a protocolului de imunohistochimie

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pentru anticorpul IMP3 (clona 69.1, DAKO). Materiale și metode: Am selectat pentru studiul imunohistochimic diverse tipuri de tumori maligne, adiacent tumorii regăsindu-se întotdeauna țesut non-tumoral (ovar, testicul, colon, pancreas, tiroidă, plămân, suprarenală). Au fost evaluate de asemenea țesuturi embrionare (provenind de la doi embrioni cu vârsta gestațională de 12 și respectiv 7 săptămâni) și placentă. Reacția de imunohistochimie a fost realizată conform procedurilor standard, după punerea la punct a protocolului de imunohistochimie, utilizând țesut ovarian normal, cu pozitivitate cunoscută pentru IMP3. Rezultate: Studiul nostru a demonstrat expresia intensă și difuză a IMP3-ului la nivelul țesuturilor embrionare somatice, placentei, precum și în unele tumori maligne. Cu toate acestea, imunomarcajul cu IMP3 a fost negativ în țesuturile normale adulte, cu excepția ovocitelor. Carcinomul tiroidian anaplastic a prezentat de asemenea pozitivitate intensă pentru IMP3, în timp ce tumorile tiroidiene bine diferențiate au fost negative pentru acest marker. Concluzii: Deoarece IMP3-ul este pozitiv doar la nivelul celulelor tumorale maligne și a țesutului embrionar, comparativ cu țesuturile non-tumorale, această moleculă ar putea reprezenta un marker prognostic foarte promițător pentru diverse tumori. IMP3-ul ar putea reprezenta și un marker cheie în identificarea tumorilor tiroidiene cu agresivitate biologică crescută. Aceasta este însă doar o ipoteză și alte studii, pe un număr mare de cazuri, cu urmărirea îndelungată a evoluției sunt necesare.

Cuvinte cheie: IMP3, imunohistochimie, embrion, ovar, carcinom tiroidian anaplastic

Introduction

IMP1, IMP2 and IMP3 are all members of the insulin-like growth factor-II (IGF-II) mRNA binding protein (IMP) family. RNA-binding proteins play important roles in gene transcription and consist of two RNA recognition motifs and four K homolog (KH) domains (1;2). *IMP3* gene, also known as *KOC* (KH-domain containing protein, over expressed in cancer) gene, is located on chromosome 7p11.2 and was originally identified from a pancreatic tumor in 1997 by Muller-Pillasch et al. (3).

Many studies have shown that IMP3 plays important roles in human embryogenesis (1;2;4;5); it is involved in RNA trafficking and stabilization, cell growth and cell migration. Strong expression of IMP3 has been reported in many fetal tissues, but its expression seems to be negative in normal adult tissues, excepting brain, placenta, ovary and testis (5). By contrast, strong and diffuse IMP3 expression has been detected in multiple human neoplasms, especially in carcinomas. IMP3 was initially identified in pancreatic carcinoma (3). Since then, it has been detected in many other human malignancies, including non-small cell lung carcinoma (6;7), hepatocellular (8), urothelial (9), bile-duct (10), ovarian (11) and uterine cervical carcinomas (12), testicular cancer (5), malignant melanoma (13), colon adenocar-

cinoma (14) and some sarcomas (15). In a prospective pilot study, Mueller F et al. tested the diagnostic accuracy of measuring KOC expression by RT-PCR as compared to the cytological assessments in fine needle aspiration from ascites, various cysts and cerebrospinal fluids. They found KOC expression to be a highly sensitive and specific indicator of malignancy (16).

In vitro studies have demonstrated that IMP3 also plays a pivotal role in tumor proliferation, invasion and metastasis (17). Moreover, IMP3 was found to be a prognostic biomarker in patients with renal cell carcinoma (18;19), clear cell type ovarian carcinoma (11), hepatocellular carcinoma (8), pancreatic ductal adenocarcinoma (20) and recently, in patients with poorly differentiated thyroid carcinoma (21).

Regarding thyroid carcinoma, two recent studies have evaluated the potential diagnostic role of this molecule in this type of cancer. The study of Slosar et al. (22) concluded that IMP3 could be diagnostically useful in differentiating malignant from benign thyroid tumors bearing a follicular growth pattern. Moreover, Jin L et al. (23) found increased levels of IMP3 mRNA expression in well-differentiated thyroid carcinomas (papillary and especially follicular carcinomas), compared to benign lesions (follicular adenoma and hyperplastic nodules) by qRT-PCR.

All in all, this molecule may represent a potential target for diagnostic and prognostic purposes in various human malignances.

In this study we first aimed to find the optimal immunostaining protocol for mouse monoclonal IMP3 antibody (DAKO, clone 69.1) and then to evaluate the expression of IMP3 in various normal human tissues, including embryonic tissues and placenta, and in various human malignancies.

Materials and methods

Samples from various normal adult human tissues (two samples of ovarian tissue, one sample of testicular, colon, pancreas, thyroid, skin, lung and adrenal tissue, respectively) were included in our study. Tumors derived from these tissues were also evaluated, comprising two cases of pancreatic ductal adenocarcinoma, three cases of lung adenocarcinoma, one case of lung squamous cell carcinoma, one case of serous ovarian carcinoma, one case of colon adenocarcinoma, one case of melanoma, two cases of testicular mixed germ cell tumors, three cases of differentiated thyroid carcinoma (one case of classic and one case of follicular variant of papillary carcinoma, one case of follicular carcinoma), one case of poorly differentiated thyroid carcinoma, one case of anaplastic thyroid carcinoma, one case of medullary thyroid carcinoma and one case of pheochromocytoma. The histopathological characteristics of these tumors are presented in *Table 1*.

Regarding embryonic tissues, IMP3 expression was evaluated in two whole-mounted embryos, of 12th and a 7th week old, together with their placentas obtained after legal (spontaneous) abortion.

The study was approved by the University Ethical Committee.

Immunohistochemistry

The immunohistochemical staining was performed on 4 µm thick sections using purified IMP3 antibody (mouse monoclonal DAKO, clone 69.1).

Sections from routinely processed formalin-fixed, paraffin-embedded tissue were placed

on sialinised slides, dried, deparaffinized, rehydrated in ethanol rinses and washed with tap water.

Standardization of the immunohistochemical protocol was performed on normal human ovary, with known positivity for IMP3. Microwave antigen retrieval was carried out for 15 and 20 min, respectively, in both citrate buffer (ph=6) and epitope retrieval solution (ph=9). Several primary antibody dilutions (1:100, 1:200 and 1:300, respectively), as well as incubation times (10min, 20min, 30min and 40 min, respectively) were tested. The best results served as guidelines to define an optimal immunostaining protocol, which was then applied to all the cases included in our study.

Consecutively, microwave antigen retrieval was carried out for 15 minutes, following immersion in citrate buffer (10% concentration, pH 6). The slides were then cooled and rinsed with distilled water. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (Thermo Scientific, Hydrogen Peroxide Block) and the slides were treated with a blocking protein (Thermo Scientific, Ultra V Block) to prevent non-specific staining. The sections were then incubated for 30 minutes with the primary antibody. IMP3 monoclonal mouse antibody was used at a 1:300 dilution. The immunodetection was performed using Ultravision LP detection system HRP-Polymer (Thermo Scientific) with DAB as chromogen, following the manufacturer's instructions. The sections were subsequently rinsed with distilled water and counterstained with hematoxylin.

The cells were regarded as positive for IMP3 when immunoreactivity was clearly observed in their cytoplasm or membrane. Samples of normal human ovary, with known positivity for IMP3, served as positive control. When present, germinal centers of lymph follicles, which are positive for IMP3, served as an internal positive control as well.

A positive result was considered when at least 10% of the tumor cells were positive for IMP3. The staining intensity was graded as weak (+), moderate (++) or strong (+++).

Table 1. IMP3 expression pattern in various human neoplasms

Cases	Histopathological features *	IMP3
Pancreatic ductal adenocarcinoma		
• Case no. 1	MD, pT3N0	Diffuse, +++
• Case no. 2	MD, pT3N0	Focal, +
Lung adenocarcinoma		
• Case no. 1	PD, pT2N2	Focal, +
• Case no. 2	MD, pT2N1	-
• Case no. 3	MD, pT2N0	-
Lung squamous cell carcinoma	MD, pT3N1	Diffuse, +++
Serous ovarian carcinoma	bilateral high grade of malignancy	Diffuse, +++
Colon adenocarcinoma	PD, pT4N2M1	Diffuse, +++
Melanoma	Clark level 4	+/-
Testicular mixed germ cell tumors		
• Case no. 1	YST	+++
	teratoma	+++
• Case no. 2	YST	+++
	immature teratoma	+++
Classic papillary thyroid carcinoma	pT3N0, multifocal, ETE	-
Papillary thyroid carcinoma, follicular variant	pT3N0, multifocal, ETE	-
Follicular carcinoma	pT2N0	-
Poorly differentiated thyroid carcinoma	pT3N0	Focal, +
Anaplastic thyroid carcinoma	pT4N0	Diffuse, +++
Medullary thyroid carcinoma	pT3N1, multifocal	-
Pheochromocytoma	probably malignant	Focal, +

Abbreviations: MD- moderately differentiated, PD -poorly differentiated, ETE- extra-thyroidal extension, YST- yolk sack tumor.

Staining intensity: weak (+), moderate (++) or strong (+++).

* pTNM in accordance with *TNM Classification of Malignant Tumors*, 7th edition, 2009 (see reference no. 26)

Results

Tables 1 and 2 summarize the results of the immunohistochemical expression of IMP3 in various normal and neoplastic human tissues (24).

Normal human tissues

In the ovary, oocytes of all types of ovarian follicles (primordial, primary, secondary, tertiary and even atretic ones), exhibited a strong cytoplasmic IMP3 expression. The immunoreactivity was limited to the oocytes, while ovarian stroma components stained negative (*Figures 1A and B*). By contrast, in the testis, only few sper-

matogonias were positive for IMP3 and the intensity of staining was very low.

Normal tissues from colon, thyroid, lung, adrenal and skin showed no immunoreactivity for IMP3. The exocrine pancreas was also negative for IMP3, but, interestingly, a weak positivity was observed in the Langerhans islets.

Embryonic tissues

We found a strong membranous and cytoplasmic IMP3 positivity in the syncytiotrophoblasts, cytotrophoblasts and mesenchymal cells of the villous cores of the placenta (*Figure 2A*). A moderate cytoplasmic positivity was also observed

Table 2. IMP3 expression pattern in various normal human tissues

Type of tissue	IMP3
Ovary	Oocytes, +++
Testis	Focal, only a few spermatogonias
Pancreas	Exocrine pancreas –, Langerhans islets +
Colonic mucosa	-
Lung	-
Thyroid	-
Skin	-
Adrenal	-
Embryo (12 weeks old)	Epithelial and mesenchymal tissues +++ - skeletal muscle, liver, kidney, gut +++
Embryo (7 weeks old)	Epithelial and mesenchymal tissues +++ - liver, kidney, pancreas, gut, developing brain +++
Placenta	Syncytiotrophoblasts, Cytotrophoblasts, mesenchymal cells in the villous core +++ Decidual cells ++

Staining intensity: weak (+), moderate (++) or strong (+++).

in decidual cells. In somatic embryonic tissues (*Figure 2B*), IMP3 had a marked granular cytoplasmic positivity in the liver (*Figure 2C*), kidney (*Figure 2D*), primitive intestinal epithelium, pancreatic acinar cells, developing brain, skeletal muscle tissue, young mesenchymal cells and epidermis. The epithelium covering the choroid plexus was also strongly positive. A moderate to weak positivity was also ob-

served in the primitive myocardial cells and bronchial epithelium.

Neoplastic human tissues

Several malignant tumors showed strong and diffuse IMP3 expression: pancreatic ductal adenocarcinoma (*Figure 3A*), lung squamous car-

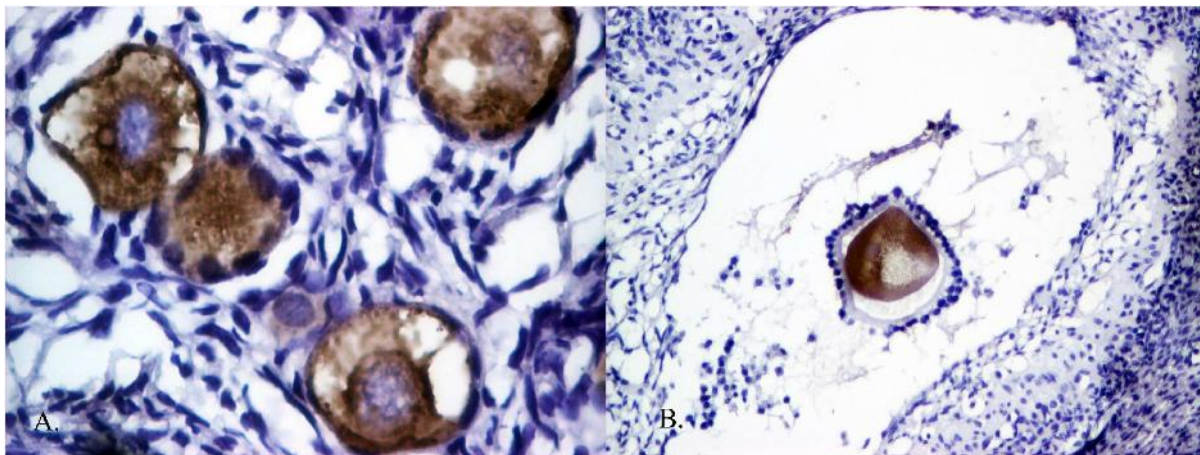


Figure 1. Ovary. IMP3 expression is positive in the primary and primordial follicles, while the ovarian stroma stains negative (**A**). Similarly, in an atretic follicle, IMP3 positivity is limited to the cytoplasm of the oocytes, while the other structures of the follicle are negative (**B**).

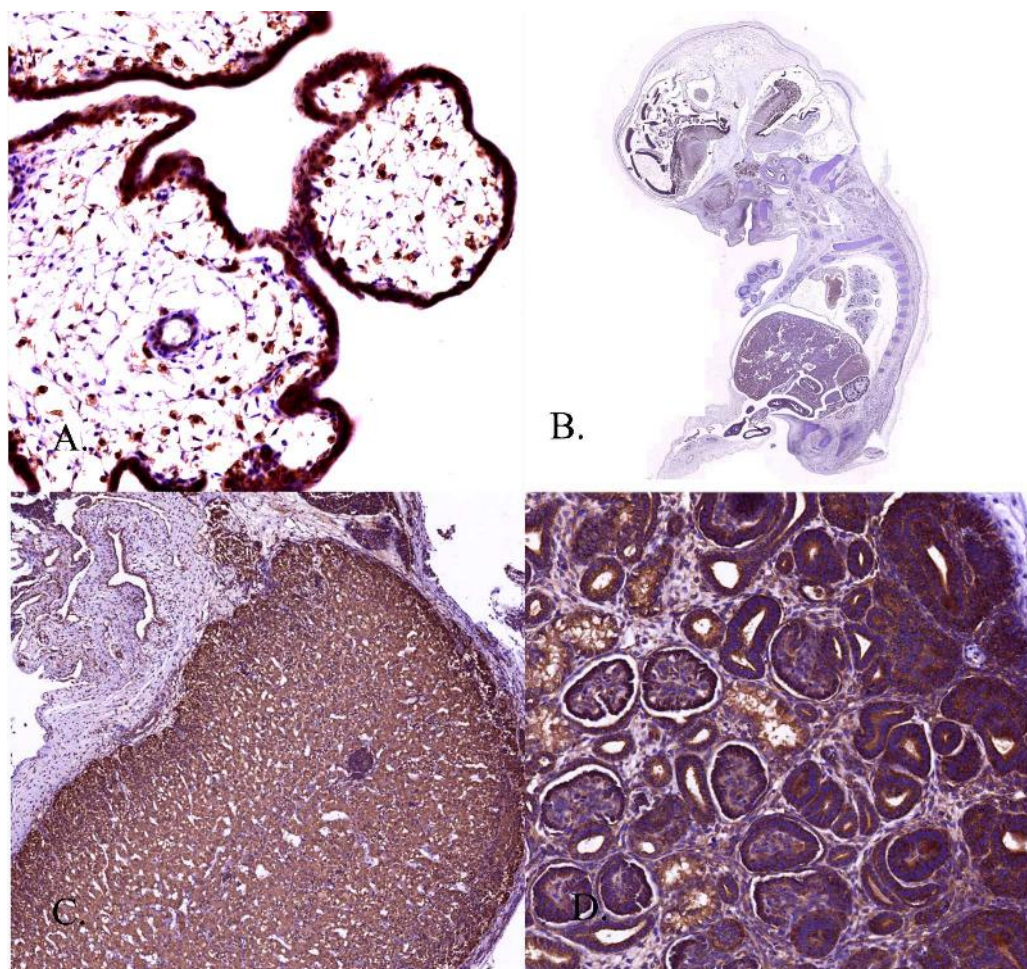


Figure 2. Placenta and embryonic tissues. Strong membranous and cytoplasmatic IMP3 positivity in syncytiotrophoblasts, cytotrophoblasts and the mesenchymal cells of the villous cores (A); 7th week old embryo, strong IMP3 immunostaining in all epithelial and mesenchymal tissues (B); 12th week old embryo, strong IMP3 expression in the liver (C) and kidney (D).

cinoma, colon adenocarcinoma, ovarian serous carcinoma and testicular germ cell tumors. Poorly differentiated lung adenocarcinoma revealed only a focal and weak IMP3 positivity, while moderately differentiated adenocarcinoma stained negative.

Regarding thyroid tumors, IMP3 expression was negative in well-differentiated follicular cell derived carcinomas (papillary and follicular carcinomas), weakly and focally positive in poorly differentiated and strongly positive in anaplastic carcinoma (Figure 3B). Medullary thyroid carcinoma did not express IMP3.

Discussion

In this study, we evaluated the expression of IMP3, an oncofetal RNA-binding protein, in various normal and neoplastic human tissues. We found that IMP3 is overexpressed in many somatic embryonic tissues, placenta, several malignant tumors and its level is undetectable in normal human adult tissues, excepting the oocytes in the ovary.

IMP1, IMP2 and IMP3 are all members of the IGF-II RNA-binding proteins family and their role in cancerogenesis and tumor progres-

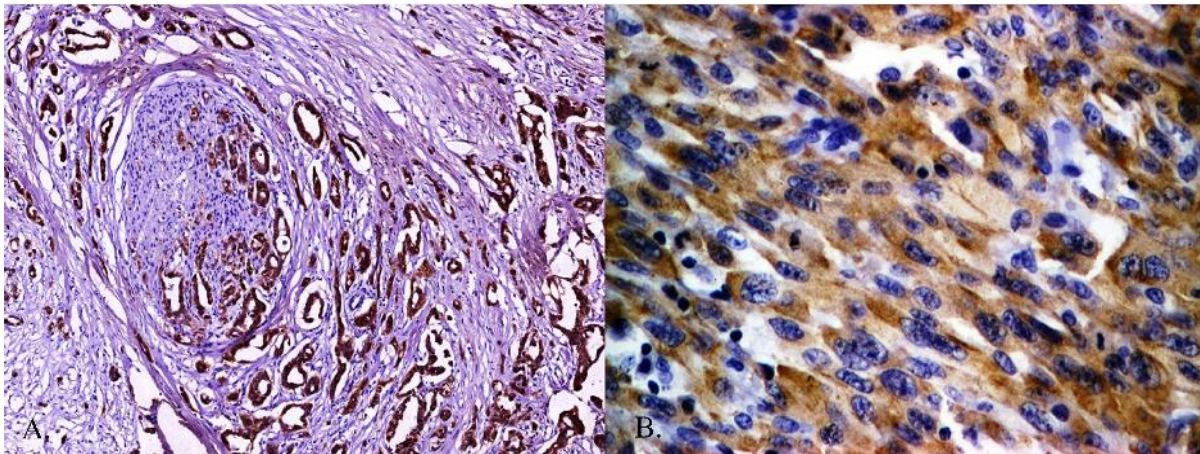


Figure 3. Malignant tumors. Strong and diffuse IMP3 expression in pancreatic ductal adenocarcinoma, with neural invasion (A) and in anaplastic thyroid carcinoma (B).

sion has largely been studied. *In vitro* studies have evaluated the functions of these molecules in HeLa adenocarcinoma cells and have demonstrated their importance in promoting cell adhesion and invadopodia formation (17). Invadopodia was described as a structure specific to metastatic tumor cells, resembling the podosomes. They are located under the cell-substratum interface and are composed of an actin-rich core that contains a variety of proteins, such as $\beta 1$ integrin, multiple signaling molecules and matrix metalloproteinases. Invadopodia generally extend into the extracellular matrix and the degradation of the matrix surrounding invadopodia correlates with the invasive potential of a cell. In this way, promoting invadopodia formation, IMPs increase the invasive capacity of malignant cells and promote cancer spreading.

IMP3 is also closely related to Vg1-RBP (Vg1 RNA binding protein) and has similar functional roles with this protein that has largely been studied in the *Xenopus laevis*' development, where it has been demonstrated to play important roles. Yaniv et al (25) reported that Vg1-RBP is required for the migration of the cells involved in the formation of the roof plate of the neural tube and in the neural crest migration. Thus, Vg1-RBP has an important role in promoting cell migration

and this might explain why IMP3 is associated with tumor progression and metastasis.

Similar to previously reported data, our study has demonstrated strong early, expression of IMP3 in the majority of embryonic epithelial and mesenchymal tissues. Endoderm-derived tissues such as liver, pancreas and gut were strongly positive. Mesodermal tissues like kidney or skeletal muscle, and ectodermal tissue as developing brain or neuroepithelium stained also positive. Extraembryonal tissues, such as placenta, showed also strong, positive expression. Consequently, it was not a surprise the intense positivity of IMP3 in yolk sack tumors and immature teratomas of the testis, tumors that partially reproduce the human embryo.

Mueller et al. have studied the organ specific expression of IMP3 (KOC) in the developing mouse and they have shown that this marker exhibits a remarkable temporal expression pattern during embryonic development. In his study, the expression of KOC seemed to be ubiquitous at early stages of embryogenesis. During advanced gestation, it was overexpressed in derivatives of all germ layers (including intestine, thymus, pancreas, kidney and developing brain). During late gestation, its expression was restricted to the deep areas of in-

testinal crypts and thymus. On the second post-natal day, IMP3 expression was no longer detectable in the epithelium of the gut (4). In our study, only two embryos of similar gestational ages were examined (a 12th and a 7th week old embryo) and intense expression of IMP3 was observed in the derivatives of all germinal cell layers (liver, pancreas, kidney, skeletal muscles, developing brain, etc). In the future, it would be of great interest to include a larger number of embryos, at different gestational ages, to study the temporal pattern of IMP3 in various organs at different moments during embryogenesis.

Although the fetal expression is prominent, data indicating that IMP3 is also present in some normal adult human tissues do exist. Hammer et al. demonstrated increased IMP3 mRNA levels in normal brain, testis and ovaries by RT-PCR (5). In our study, IMP3 stained positive in the cytoplasm of both resting and growing oocytes. A focal, weak positivity was also observed in a few spermatogonias. We also found a weak positivity in the Langerhans islets of the pancreas, a very interesting result since the origin of these structures, derived from the neural crest is very different from the acinar cells, which are endoderm-derived. However, IMP3 expression was negative in medullary thyroid carcinoma and only a few cells stained positive in pheochromocytoma.

We found strong, diffuse IMP3 expression in several malignant tumors. Both ductal pancreatic adenocarcinomas stained positively for IMP3, with a strong, diffuse positivity in one case and a weak, focal expression in the latter. Mueller-Pillasch et al. first demonstrated increased KOC expression in pancreatic cancer cells as compared to both normal pancreas and chronic pancreatitis tissue and consecutively described IMP3 as the over-expressed K-homologue domain containing protein (KOC) (3). KH-domain containing proteins have important functions in the regulation of mRNA stability (26), which is implicated in fundamental biological processes such as development, cell

growth, differentiation and carcinogenesis. Based on the nature of proteins containing KH domain and the expression patterns observed in their study, Mueller et al. suggested that KOC may play a role in the regulation of tumor cell proliferation by interfering with transcriptional or posttranscriptional processes (3).

IMP3 expression has previously been demonstrated to be a prognostic marker in pancreatic carcinoma (20), renal cell carcinoma (18;19), and recently, IMP3 expression was found to be correlated with the histologic grade in lung adenocarcinoma (7). In our study, we found a weak, focal IMP3 positivity in poorly differentiated lung adenocarcinoma, while moderately differentiated lung adenocarcinoma cases stained negative.

Regarding thyroid carcinoma, in our study, IMP3 expression was negative in well-differentiated thyroid tumors (papillary and follicular carcinomas), weakly and focally positive in poorly differentiated carcinoma and strongly positive in anaplastic carcinoma. These results may suggest that IMP3 can be used as a prognostic marker of thyroid carcinoma, since it is mainly positive in tumors known to have increased biologic aggressiveness. However, this is only a hypothesis and the prognostic significance of IMP3 in thyroid carcinoma needs to be further studied on larger number of cases, with long-term follow-up. Recently, a large multicenter study has identified IMP3 as a predictor of poor prognosis in poorly differentiated carcinoma. Their results showed that IMP3 positivity was associated with an increase risk of death, lymph node and distant metastases and a decrease of disease-free survival (21).

However, the biologic significance of IMP3 overexpression in thyroid carcinoma is so far unclear. Two other recent studies have evaluated the role of IMP3 as a molecular marker to assist the diagnosis of thyroid carcinoma. The study performed by Sloasar et al. (22) showed a 100% specificity and a 100% positive predictive value for IMP3 in differentiating between

follicular carcinoma and follicular adenoma, by immunohistochemical assay. Later on, Jin et al. (23) showed that measuring IMP3 by qRT-PCR is more sensitive than immunohistochemistry and IMP3 mRNA was found to be overexpressed in well-differentiated thyroid carcinoma compared to benign thyroid lesions. By contrast, in our study, we found a negative IMP3 expression in both papillary and follicular thyroid carcinoma. However, no conclusion can be drawn since only a few cases were evaluated by us and only by immunohistochemistry. In the future, our goal is to assess the value of IMP3 as a diagnostic tool in various types of thyroid lesions.

Conclusion

To our knowledge, this is the first study performed in Romania to evaluate IMP3 immunohistochemical expression in various normal and neoplastic human tissues, including embryonic tissues and placenta. We found that IMP3 is overexpressed in many somatic embryonic tissues, in placenta, some malignant tumors and its level is undetectable in the great majority of normal human tissues, excepting the oocytes of the ovary. Since IMP3 was positive in embryonic and tumor tissue only, but not in normal cells, this molecule might represent a very promising marker in the prognostic evaluation of various tumors.

Our findings regarding thyroid carcinoma, strong and diffuse expression of IMP3 in anaplastic thyroid carcinoma and negative staining in differentiated tumors, might suggest that IMP3 could also be used as a prognostic marker in thyroid carcinoma. However, this is only a hypothesis and further studies on larger number of cases, with long-term follow-up are needed to test the prognostic significance of IMP3 in thyroid carcinoma.

Conflict of interest statement

None declared.

Abbreviations

IMP - insulin-like growth factor-II (IGF-II) mRNA binding protein
KH - K homolog
KOC - KH-domain containing protein, over expressed in cancer

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