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DOCTORAL THESIS
VASCULAR TUMOURS IN CHILDREN.
REFINEMENT OF THE DIAGNOSIS WITH
THERAPEUTIC IMPACT IN THE
CLINICOPATHOLOGICAL AND EXPERIMENTAL
MODEL
-ABSTRACT-

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Key words: hemangioma, vascular tumours, endothelial cell, molecular profile, infantile.

INTRODUCTION

The classification of vascular anomalies was unclear for a long time; the term "angioma" was used incorrectly, being employed both for vascular tumours and for vascular malformations. Unfortunately, the discrepancies between the clinical and histopathological classifications persist. There is a great diversity of vascular tumours in children; many of them are benign, limited, some may have a local aggressive character or may recur after excision. The lesions with a low degree of malignancy, potential for metastasis or fatal evolution occur less frequently.

Infantile hemangiomas appear between 2 weeks and 2 months of age, and have a proliferative growth phase until they reach their full size, while congenital hemangiomas are distinguished by the fact that they are present in their full form at birth. Hepatic infantile hemangiomas, which are pathologically identical to those located on the skin or other organs, have been considered infantile hemangioendotheliomas, mistakenly suggesting that they have a distinct pathology. Histological studies have shown that the lesion known so far as "hemangioma" of the liver and of the vertebrae in adults, as well as the orbital cavernous hemangioma, are venous malformations rather than neoplasms.

It is well-known that infantile hemangioma is the most notorious tumour of childhood (5-10% incidence), consisting of endothelial cell proliferation and pericytes. The evolution of hemangiomas is unpredictable, most of them regress spontaneously, while only about 10% are destructive, disfiguring and endanger the patient's life; the factors determining the progression, regression and heterogeneity of the response to conventional therapy are not fully known. All these are due to the lack of personalized molecular profiling for each type of hemangioma.

Several studies prove the usefulness of the ISSVA classification, due to the frequent confusions regarding the diagnosis and treatment of hemangiomas. All these are due to the widespread use of the term "hemangioma" for a large number of vascular lesions. Recent studies have provided some data on the pathogenesis of these vascular tumours, resulting into a better understanding of neovascularisation in the evolution of the hemangioma, and highlighting two main pathogenic mechanisms: angiogenesis and vasculogenesis. There are studies that have tried to create an angiogenic profile of the endothelial cell in hemangiomas, but so far, in practice, the implementation of a targeted therapy has not been possible.

MOTIVATION

Infantile and congenital hemangiomas still represent a challenge for both the paediatric surgeon and the paediatrician. The paediatrician establishes the treatment for infantile hemangiomas completely randomly, only based on the anamnesis, but also on the location of the hemangioma. Most often, hemangiomas appearing in the areas where the extension capacity is limited are treated by surgery, so as to avoid endangering the patient's life. At present, regardless of the application method, the results obtained are controversial, which is why, except for surgery, no other treatment is currently standardized in infantile and congenital hemangiomas. Therefore, many other adjuvant therapies have been tested.

Multiple criteria for evaluating the propranolol treatment are tested in paediatric practice starting from the recommended dose up to the regression mode or age at which it can be administered.

The histopathological classification of hemangiomas has been recently revised, but recurrences following discontinuation of the adjuvant or combination therapies could not be explained. A molecular classification of hemangiomas is practically non-existent at the present time, and, consequently a targeted therapy of infantile and congenital hemangiomas is not currently possible. Another controversial and unsolved aspect, which is therefore unexploited from a therapeutic point of view, is represented by the expression and role of lymphatic markers in infantile and congenital hemangiomas. Apparently, the expression of lymphatic markers is variable, and it depends on the topography of hemangiomas, but also on the evolutionary stage.

The literature does not include any data regarding the different gene expression in the VEGF axis between treated and untreated hemangiomas. Based on the arguments above, this study proposes the following objectives:

1. The statistical analysis of infantile and congenital hemangiomas in the western part of the country.
2. The expression of blood and lymphatic vascular markers in primary and recurrent infantile and congenital hemangiomas.
3. The role of Prox1, PDGF-BB, CLIC1 with respect to the GLUT1 expression well studied in infantile hemangiomas for the identification of new prognostic and therapeutic factors.
4. The protein and molecular evaluation of the VEGF axis gene expression in involuting versus proliferative hemangiomas
5. The immunophenotyping of hemangiomas with particular location, rarely encountered in medical practice.

GENERAL PART

Vascular birthmarks were described over time, and many famous figures presented vascular anomalies. The first description of a vascular anomaly appears in 460 BC, when an arterial aneurysm is discovered by Hippocrates.

During the 1950s and 1960s, an important step was made in the field of endothelial cells by electron microscopy. The presence of specific organs such as the Weibel-Palade corpuscles or the existence of plasmalemmal vesicles, later called caveolae, are just some of the ultrastructural aspects described at that time.

Over time, there have been several classifications of vascular anomalies, some of which have failed to establish a correlation between the clinical and the histological classification, which is why a series of confusions have arisen. The accuracy of the diagnosis is very important for the evaluation, and management, and, sometimes, diagnosis is interdisciplinary, as it involves a close collaboration between surgical and medical specialties.

Usually, the reference classification of tumours or tumoral-like lesions is the one made by the World Health Organization (WHO), but in the case of vascular lesions the classification and nomenclature include inconsistencies. Starting from the Mulliken and Glowacki classification, ISSVA introduced and adopted, at the meeting held in Rome in June 1996, the binary “biological” classification.

Imaging investigations are used in vascular tumours to demonstrate visceral involvement, schedule the surgical excision, evaluate the effectiveness of treatment, and/or highlight the potential associated lesions.

The study of vascular anomalies has led to the identification of genes and genetic pattern, which, in turn, play an important role in the development of specific therapies.

Different treatment methods were documented for infantile hemangiomas, including waiting and monitoring the lesion, laser therapy, drug therapy, sclerotherapy, radiation therapy, surgery, and so on, but none of these therapies can be used for all hemangiomas. In order to obtain the best treatment results, the treatment protocol must be individualized and comprehensive, as well as sequential.

The treatment of hemangiomas must be individualized depending on: the type of lesion, location, size, depth, stage of growth and evolution of the lesion. So far, none of the available treatments is considered standard therapy. This aspect is a starting point for the identification of new specific therapeutic targets that would preserve normal endothelial cells and determine the

regression of the hemangioma, especially of the recurrent ones and of those with an increased proliferation rate.

SPECIAL PART

50 cases have been evaluated in the current study. The bioptic fragments were taken by surgery from patients hospitalized at "Louis Turcanu" Emergency Clinical Hospital for Children in Timisoara between 2014 and 2019. The tissue fragments fell within the limits of the standard dimensions, having less than 1cm³. The sampling was followed by fragment fixation and processing, and, at the end, the paraffin blocks were sectioned and prepared for staining. The evaluation of the morphologically stained materials was followed by case selection for the histochemical and immunohistochemical staining procedures.

EPIDEMIOLOGICAL, CLINICAL AND STATISTICAL CHARACTERIZATION OF THE CASES INCLUDED IN THE STUDY

We have studied 50 vascular anomalies in hospitalized children aged 2 months to 17 years. The studied cases are divided, according to the ISSVA 2014 classification, into vascular tumours (41 cases) and simple vascular malformations (9 cases). Vascular tumours are represented by hemangiomas (33 cases), glomangiomas (2 cases), pyogenic granulomas (5 cases) and one kaposiform hemangioendothelioma.

Vascular tumours are most frequently located in the trunk and head or neck area, while arteriovenous malformations are common at the limb level.

Depending on the histopathological characteristics and intensity of the PROX1 expression, infantile hemangiomas were divided into four categories: hemangiomas in the proliferative phase, hemangiomas in the early involution phase, hemangiomas in the advanced involution phase and involuted hemangiomas.

The intensity of the PROX1 reaction is maximum in the involuted and muscular hemangiomas, and it is negative in the proliferative-phase hemangiomas.

The expression of PDGF B is variable in infantile hemangiomas; thus, in the proliferative phase, for most of them, the reaction intensity is 1 (5 cases), and for only one case the intensity is 3, as it probably is in a delayed proliferative phase. The reaction intensity is variable, as the hemangioma develops, and most of the involuted ones present a maximum intensity of the

reaction. A maximum intensity of the PDGF B reaction is also identified in the case of arteriovenous malformations.

Among the cases we studied, there were two cases of treated hemangiomas. For the one treated with propranolol, the PROX reaction intensity was 3; this was considered an involuting hemangioma. The second case was a proliferative hemangioma, treated with propranolol (without any results, it grew and ulcerated), which was surgically excised and cauterized; afterwards it relapsed, with a negative response to PROX1.

Out of the 50 cases we have studied, most were identified in females (25 cases). The lymphangioma and intramuscular hemangioma were found in the female population, and the pyogenic granulomas in boys.

The hemangiomas in the proliferative and incipient involution phase were diagnosed in children aged between 2 months and two years, and most of them were identified in the first year of life. Modifications corresponding to a hemangioma in the advanced involution phase or to an involuted hemangioma appear from the first year of life, and were found up to the age of 12. The youngest patient who was diagnosed with intramuscular hemangioma was 4 years old, and the oldest was 17. The arteriovenous malformation was identified in children over 2 years of age.

MOLECULAR CHARACTERIZATION OF THE RARE FORMS OF VASCULAR MALFORMATIONS

Rare vascular tumours such as the intestinal lymphangioma or thymic hemangioma are rarely reported in the literature and even less evaluated in terms of immunohistochemical and molecular phenotype. We considered it useful to insert in the study on hemangiomas carried out in this PhD thesis two rare tumours such as the intestinal lymphangioma and thymic hemangioma, with special attention not to the reporting as such, but to the immunophenotyping of the two types of tumours, mainly focusing on the growth factors and their corresponding receptors involved in the angiogenesis and lymphangiogenesis processes, aspects that are little studied with respect to vascular tumours, and even less concerning their forms with rare location.

The data we have obtained demonstrated the existence of an active angiogenic and lymphangiogenic process, both by the expression of the VEGF and PDGF growth factors, and by the morphological aspects observed, as well as by the expression of the Prox1 transcription factors present in both types of vascular tumours.

INTESTINAL LYMPHANGIOMA: A VASCULAR MALFORMATION OR TUMOUR INCOMPLETELY CHARACTERIZED FROM THE MOLECULAR POINT OF VIEW

We presented a case of multicystic mesenteric lymphangioma, a vascular entity extremely rare in children and extremely versatile in terms of clinical diagnosis in the absence of imaging evaluations, as it can be easily confused with acute appendicitis. The patient's normal genetic profile has forced us to critically discuss other hypotheses regarding the origin of lymphangiomas, LEC status, role of Prox1 in the pathogenesis of lymphangiomas, as well as to review the literature on VEGFR 3 and PDGFR beta on such lesions, as they are most likely to be addressed in the future as potential specific therapeutic targets in the treatment of lymphangiomas.

The results we have obtained demonstrated the existence of immature and activated LECs, given that most of them expressed Prox1. Concerning our case and correlated to a high proliferation index that we noticed in the lymphatic endothelium of the hemangioma, we can conclude that this vascular malformation (tumour) is an active lesion containing activated LECs that could be a source of cells which may be responsible for the recurrences described for these lymphangiomas, although such recurrences are indeed sporadic. For this reason, we consider that Prox1 in combination with Ki-67 should be used for the routine evaluation of lymphangiomas, as their expression can be considered a suggestive prognostic factor for possible recurrences.

VASCULAR MALFORMATIONS LOCATED IN THE THYMUS

Vascular malformations located in the thymus represent a group of rare vascular tumours, the literature currently mentioning only 16 cases worldwide.

A particular aspect was the involvement of the thymic stromal cells in the pathogenesis of the thymic hemangioma. We noticed that the number of CK-positive thymic stromal cells decreased significantly, inversely proportional to the number of pseudovascular structures of the hemangioma; the distribution, morphology and interrelationship with the surrounding capillary structures of the thymic stromal epithelial cells were highly suggestive of the transdifferentiation process by which the thymic epithelial cells turn into endothelial cells capable of lining the pseudovascular structures within the thymic hemangioma.

This thesis is the first to mention the involvement of lymphatic vessels in thymic hemangiomas, as well as the dynamics of the two markers studied,

i.e. the differentiation marker towards the lymphatic line (Prox1), and the identification marker for the differentiated lymphatic vessels (D2-40). Their role in thymic hemangiomas is not currently certified in the literature.

CONCLUSIONS

The identification of the expression of lymphangiogenic markers in the intestinal lymphangiomas or lymphangiomas with a different localization opens up the prospect of using targeted therapies in order to avoid surgical therapies if they are not strictly necessary for removing these malformations.

1. This study supports the application of an extensive evaluation of vascular malformations in children, as a basis for a future molecular classification which should include prognostic and therapeutic factors, as well as predictive factors for possible relapses.
2. The evaluation of the growth factors in the VEGF and PDGF family must be part of the test battery for the initial evaluation of hemangiomas, given that they are the two main signalling pathways.
3. The CD34/CK-HMW double immunostaining proved there is a decrease in the number of stromal epithelial cells directly proportional to the increase in the number of capillary structures of the tumour formation.
4. Our data support the possibility for stromal epithelial cells to transdifferentiate into endothelial cells, and to actively participate in the angiogenesis process of such lesions.
5. The thymic hemangioma contains cells with the ability to differentiate towards the lymphatic line, an aspect not reported in the literature up to this date. A significant difference was identified in the expression of Prox1 between the differentiated and poorly differentiated areas, as well as between the number of Prox1-positive cells and the lymphatic vascular microdensity detected with D2-40.

GENE EXPRESSION DIFFERENCES OF THE VEGF PATHWAY EVALUATED USING THE TAQMAN TECHNIQUE BETWEEN PROLIFERATIVE INFANTILE HEMANGIOMAS AND INVOLUTING INFANTILE HEMANGIOMAS

The propranolol therapy is widely used for treating infantile hemangiomas, but with variable results and, sometimes, with the occurrence of

post-treatment relapses. The hypothesis of an exaggerated angiogenesis as the first pathogenic mechanism of hemangioma production is increasingly mentioned.

We considered it was important to evaluate 30 of the 40 genes included in the TaqMan Array kit. The gene amplification of VEGF A and VEGF C was present in both samples, and gene overexpression was identified for both types of hemangiomas.

CONCLUSIONS

1. The gene analysis of the VEGF pathway using the TaqMan Array technique was applied comparatively for involuting and proliferative infantile hemangiomas to identify unfavourable prognostic factors and recurrence predictive factors in the involuting hemangiomas.
2. This study identified gene amplification for 11 genes of the VEGF pathway in the involuting infantile hemangioma versus 22 genes for the proliferative infantile hemangioma. All 11 amplified genes amplified in the involuting hemangioma were also amplified in the proliferative infantile hemangioma.
3. The MAPK/AKT1 pathway remains activated in the involuting infantile hemangioma.
4. The heat-shock protein family (Hsp27 and HSp90 alpha) is also activated in the involuting hemangioma.
5. KDR was not amplified in the involuting hemangioma despite the overexpression of VEGF, which supports a possible recurrence due to other angiogenic mechanisms.
6. The MAPK pathway may be responsible for the cause of recurrences in involuting hemangiomas.
7. MAPK14, MAP2K2 and MAP2K6 were identified as the main promoters of the endothelial cell activation.
8. ACTG1 and ACTG2, together with ACTB preserved overexpression in the involuting hemangioma and can be considered as a factor favouring recurrences under certain stress conditions.
9. The amplification of ACTG1 and ACTG2 in the involuting hemangiomas also suggests the existence of a stem cell pole with an increased reactivation capacity, which makes the epithelial-mesenchymal transition possible.
10. Two genes have been identified for the first time as overexpressed in the involuting hemangioma, i.e. HSP90 alpha and BAD. Their role in

the involuting and proliferative hemangiomas has not been reported in the literature so far.

8. STUDY OF THE INTERRELATION BETWEEN GLUT1 AND OTHER PROGNOSTIC MARKERS IN INFANTILE VASCULAR MALFORMATIONS

Numerous published articles currently suggest that it is necessary to make a molecular classification of congenital and infantile hemangiomas by studying new markers with prognostic and therapeutic potential. The glucose transporter 1 (GLUT1) is a marker used in the study of hemangiomas, being positive in over 95% of the infantile hemangiomas, and negative in congenital hemangiomas.

The platelet-derived growth factor (PDGF) is little studied in infantile vascular malformations. Concerning CLIC1, the protein associated with chlorine channels, no data is currently available on its involvement in the pathogenesis of infantile or congenital hemangiomas. The inhibition of chlorine channels by the application of chlorotoxin determines an intense antiangiogenic effect.

We evaluated the positive expressions of CLIC1, GLUT1, PDGF-BB, Prox1. For CLIC1 and GLUT1, we quantified the nuclear, cytoplasmic and combined expressions. For PDGF-BB, the interpreted expression was the cytoplasmic one. For GLUT1 and CLIC1, the area that was positive to these markers was also quantified in relation to the total analyzed section. We also wanted to see if these markers are influenced by sex or by the type of hemangioma, i.e. proliferative versus involuting. Moreover, we wanted to observe the expression variability of these markers depending on their location.

A particular aspect that we encountered in our study, and that is not reported in the literature, refers to the nuclear expression of GLUT1, as well as to the mixed, nuclear and cytoplasmic expression. 18% of the total hemangiomas studied showed a nuclear expression in GLUT 1, but all of them had an associated cytoplasmic expression.

The CLIC1/GLUT1 coexpression was also analyzed in the proliferative hemangiomas versus involuting hemangiomas. In the proliferative hemangiomas, the correlation of the GLUT1-positive area with the CLIC1-positive area was statistically significant.

The correlation of the CLIC1-positive area with age, sex or location of the hemangiomas was not statistically significant. On the other hand, the

correlation of the GLUT1-positive area in proliferative hemangiomas with the patients' sex was statistically significant, the 100% positive areas being predominantly represented in the female patients. This aspect was quantified in the cases studied based on the literature data that suggest a preponderance of the CLIC1 expression in males, which we also certified in the proliferative hemangiomas, given that the nuclear expression of CLIC1 is considered an unfavourable prognostic factor in malignant pathologies. Moreover, the combined nuclear and cytoplasmic expression was specific to male patients.

It is worth mentioning that all three types of CLIC1 expression were influenced by the age of the patients with hemangiomas. The correlation of the CLIC1/C and GLUT1/C expressions was statistically significant in the proliferative hemangiomas of the head and neck.

At the thorax level, all proliferative hemangiomas showed a positive reaction to GLUT1 in the entire tumour area. Compared to the proliferative hemangiomas of the head and neck, thoracic hemangiomas seem to be much more influenced by the GLUT1/CLIC1 tandem.

It is interesting to note the complete absence of the PROX1 immunoreaction in all cases of proliferative hemangiomas with thoracic location, as well as the intense immunoexpression of GLUT1 (+3) in all cases having this location.

The nuclear expression of CLIC1 was also present in most cases, except for one. This may be considered an unfavourable prognostic factor for proliferative hemangiomas with this location.

All intramuscular hemangiomas were diagnosed in female patients, in the upper or lower limbs and, as a particular aspect, all cases showed a +3 reaction to Prox1. For these hemangiomas with muscular location we did not register any statistically significant correlation between the expression of GLUT1 and CLIC1.

Due to the above-mentioned results, we considered it useful to study the expression of the markers included, depending on the location of hemangiomas, i.e. in the head and neck area, in the thorax or other locations. Concerning the involuting hemangiomas of the head and neck, PDGF-BB seems to play an important role in their evolution. Thus, the correlation of PDGF-BB was statistically significant with both PROX1 and GLUT1/C.