

ORIGINAL PAPER

Diagnostic and clinical significance of D2-40 expression in the normal human thymus and thymoma

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Abstract

Human thymus development and thymoma behavior remain elusive, in spite of many acquisitions in the field in last decades. In the present paper, we analyzed the immunohistochemical expression of D2-40 in the normal human thymus and thymoma. In both fetal and postnatal normal thymus, we found a strong expression of D2-40 in the subcapsular and cortico-medullary epithelial cells, and lack of expression in the thymus of involution. These findings support a role for podoplanin in the proliferation of some subtypes of epithelial cells of the normal thymus stroma. In thymoma, the expression of D2-40 was detected in neoplastic cells in 18 from 26 cases (69.23%). No correlation was found between D2-40 expression and histological types of thymoma, but strong correlation was noticed with tumor stage. Based on these results, it is suggested that D2-40 expression is a good predictor of invasion and can be considered as a potential target for therapy in selected cases.

Keywords: thymus, thymoma, immunohistochemistry, D2-40, prognosis.

✉ Introduction

Thymus has a crucial role in the development of the immune system of the organism. This main function is accomplished by the particular microenvironment of the organ that finally leads to the maturation of T cells and stimulates differentiation of the other lymphoid organs. Although the development of the thymus was extensively investigated in experimental studies, relatively few data are available about the organogenesis of the normal human thymus [1, 2]. The epithelial stroma of the thymus is endodermal in origin and consists of many subtypes of highly specialized cells [3, 4]. On the other hand, factors that stimulate proliferation of epithelial cells during development and maintenance of the cell population in the postnatal life are less understood. There were accumulated many data about the gene control of epithelial cell proliferation in experimental models and in rodents there were characterized thymus epithelial stem cells [5]. However, the investigation of the multi-step process of thymus stroma differentiation at protein level in the normal human thymus was not performed.

The epithelial cells of the thymus stroma support the development of the organ-specific tumors of the thymus, known as thymomas. Thymoma is a relatively rare neoplastic condition with unpredictable behavior and few molecular factors were shown to be useful to predict the outcome of the patient [6, 7]. Based on these

data, in the present moment it seems that the most important prognostic factor is the tumor stage and the main role of the pathologist is to discriminate between true thymoma and thymus carcinoma. Some molecules, like CD117 and CD5 have been shown useful for this purpose [8, 9]. Moreover, the histological classification of thymoma, as accepted by the *World Health Organization* [10] seems to be one of the most elusive from all classifications of human tumors.

D2-40 recognizes the formalin-insensitive epitope of podoplanin and nowadays is the most used antibodies to demonstrate lymphatic endothelium. D2-40 have been raised against the oncofetal antigen M2A, associated with germ cell neoplasia, fetal gonocytes, and re-expressed in germ cell tumors [11]. Double immunostaining based on anti-D2-40 and anti-CD34 demonstrated that the final product of reaction is restricted to the lymphatic endothelium and does not stain blood vessels' endothelium in both normal and tumor tissues [12, 13]. The expression of podoplanin is regulated by the lymphatic-specific homeobox gene *Prox1*, a master gene that controls the development of lymphatic progenitors from embryonic veins [14].

Although very useful to differentiate between lymphatics and blood vessels, D2-40 expression is not completely specific for the lymphatic endothelium. D2-40 expression was reported in a relatively large spectrum of human tumors, like angiosarcoma [15], mesothelioma [16], germ cell tumors [17], squamous

cell carcinoma [18] or some subtypes of brain tumors [19]. At present time, there are no data regarding the expression of D2-40 in thymoma, and virtually, its clinical significance in this condition is unknown.

In the present study, we show an early expression of D2-40 during human thymus development and a particular distribution in the postnatal thymus. We found that D2-40 is differently expressed in thymomas, regardless the histological type, and based on this data, D2-40 expression could be an indicator of tumor progression.

☐ Material and Methods

There were investigated specimens of human thymus taken from five fetuses (aged between four and seven months), 11 postnatal normal thymuses (aged between one week and 12 years), 10 with thymus involution, 15 with myasthenia gravis and 26 thymomas.

The specimens of normal thymuses were tactically removed during the surgical repair of cardiovascular defects. All specimens were fixed in buffer formalin and embedded in paraffin using the routine histological procedure. Hematoxylin–Eosin stained slides were used for the pathological diagnosis.

Thymomas were classified using *WHO* criteria and we found thymoma type A in three cases, type AB in two cases, type B1 in six cases, type B2 in five cases, type B3 in seven cases, and thymic carcinoma in three cases. Based on the macroscopic and microscopic aspects, there were 17 invasive and nine non-invasive thymomas.

Additional sections, 3 μ m thick, were stained with anti-D2-40. Briefly, sections were dewaxed in benzene and hydrated in decreasing solutions of alcohols. Antigen retrieval was performed with microwave using citrate buffer pH 6 for 30 minutes. After blocking the endogenous peroxidase, slides were incubated with anti-D2-40 for 30 minutes (ready-to-use, Dako Cytomation, CA, USA). The working system was LSAB-HRP and the final product of reaction was visualized with diaminobenzidine (Dako, Glostrup, Denmark). Nuclei were stained with Lillie's modified

Hematoxylin. The entire immunohistochemical procedure was performed with DakoAutostainer Plus (Dako Cytomation, Denmark).

The statistical analysis was performed with the commercially available SPSS version 17.0. Student *t*-test and Chi-square were done to analyze the significance of D2-40 expression by tumor cells, and $p < 0.05$ was considered as significant.

☐ Results

In the fetal thymus, the immunohistochemical reaction for D2-40 was positive in all of the cases. Particularly, the distribution of the final product of reaction was dependent on the age of the fetus. In fetuses of four and five months, there were intensely stained lymphatic vessels from the surrounding connective tissue and epithelial cells of the thymus stroma located in the periphery of lobules (Figure 1a). No positive reaction was noticed in the epithelial cells of the deep cortex and only a weak to moderate reaction was found in the epithelial cells at the junction between cortex and medulla (Figure 1b).

In the 6 to 7-month-old fetuses, the subcapsular epithelial cells retained the strong positivity to D2-40, but we noticed a gradual developing of the medullary epithelial cell network (Figure 1, c and d). In the normal postnatal thymus, the positive reaction for D2-40 was restricted to the subcapsular and medullary regions of the thymic lobules and was negative in the cortex (Figure 1e). A well-developed network of strongly stained epithelial cells was found at the junction between cortex and medulla (Figure 1f).

A marked decrease in the intensity of D2-40 staining was found in the cases with thymus of involution. The reaction was negative in six from 10 cases in the medulla, and in all of the cases in the subcapsular region of the thymic lobules (not showed). The positive reaction found in four cases in the epithelial cells of the medulla was weak and heterogeneous without enhancement at the junction between cortex and medulla. Similar features were found in all the cases with myasthenia gravis.

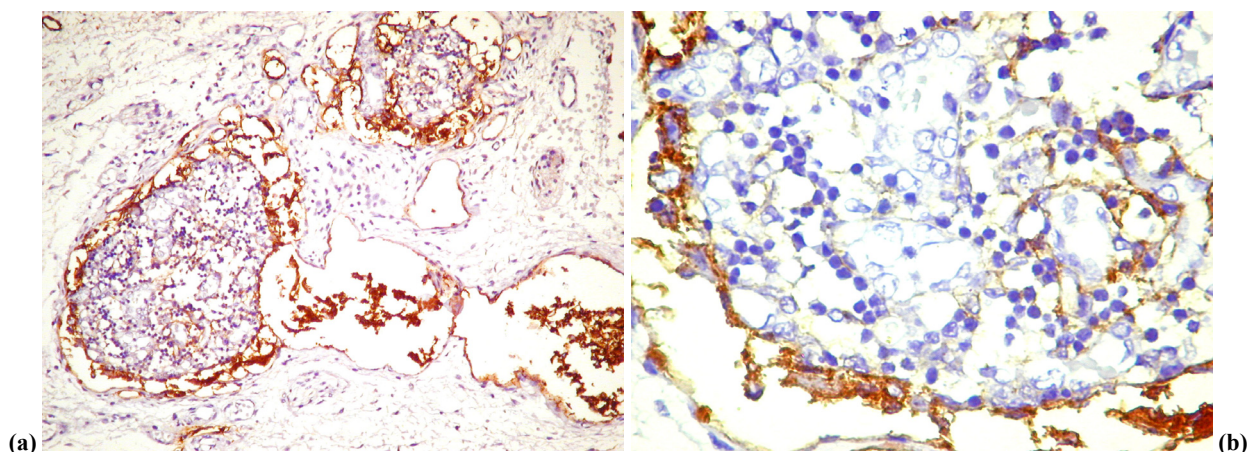


Figure 1 – Strong reaction in the lymphatic vessels of the connective tissue and epithelial cells in the periphery of thymic lobules (a, $\times 100$). Strong reaction in the subcapsular epithelial cells and weak positive reaction at the junction between cortex and medulla (b, $\times 400$).

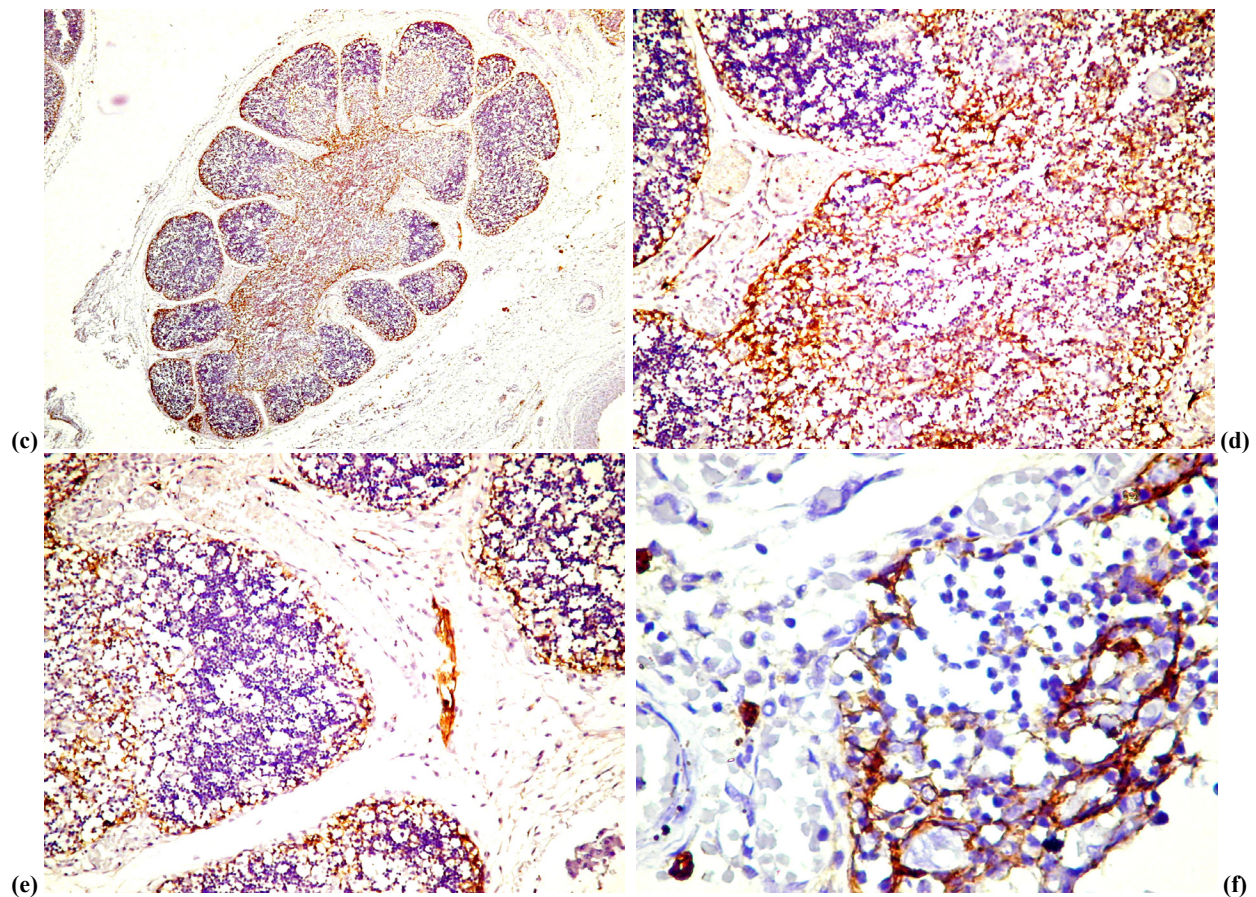


Figure 1 (continued) – Six months old fetal thymus with strong reaction for both subcapsular and medullary epithelial cells (c, $\times 4$ and d, $\times 100$). Normal postnatal thymus, positive reaction in the subcapsular and medullary epithelial cells (e, $\times 100$). The junction between cortex and medulla shows a rich network of intensely stained epithelial cells (f, $\times 400$). Anti-D2-40 staining.

Examination of the cases with thymomas focused on neoplastic cells and lymphatic vessels. Lymphatic vessels were found only in the peritumoral connective tissue and not in the tumor area. Usually in patients with thymoma lymphatics had thin wall, relatively large lumen, devoid of lymphocytes of neoplastic cells (Figure 2a). Lymphocytes were found in the lumen of the lymphatic vessels in only three cases with thymoma (Figure 2b), and lymphovascular invasion was found in only one case with thymic carcinoma (Figure 2c). No correlation was found between the lymphatic

microvascular density and the invasive character ($p=0.43$) or with the pathological type of thymoma ($p=0.21$).

In thymomas, the final product of reaction was had cytoplasmic granular pattern, with membrane enhancement. The expression of D2-40 was found in neoplastic cells of thymoma in 18 from 26 cases. From these, 15 were invasive and three non-invasive thymomas. A significant correlation was found between the invasive character of the tumor and expression of D2-40 by tumor cells ($p<0.001$).

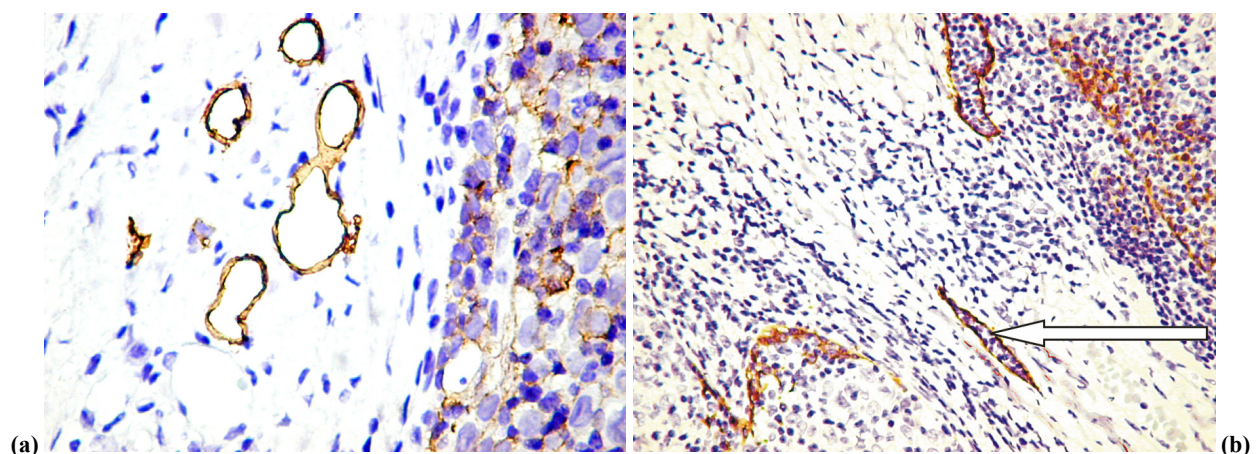


Figure 2 – Lymphatic vessels in the peritumoral tissue in a case with thymoma (a, $\times 400$). Lymphatic vessel containing lymphocytes (b, arrow, $\times 100$). Anti-D2-40 staining.

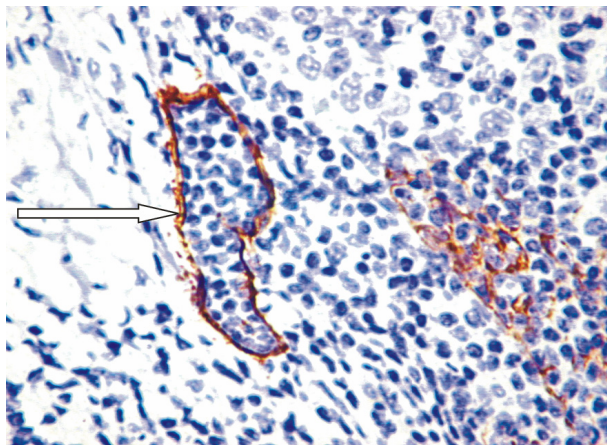


Figure 2 (continued) – Lymphatic vessel containing neoplastic cells (c, arrow, $\times 400$). Anti-D2-40 staining.

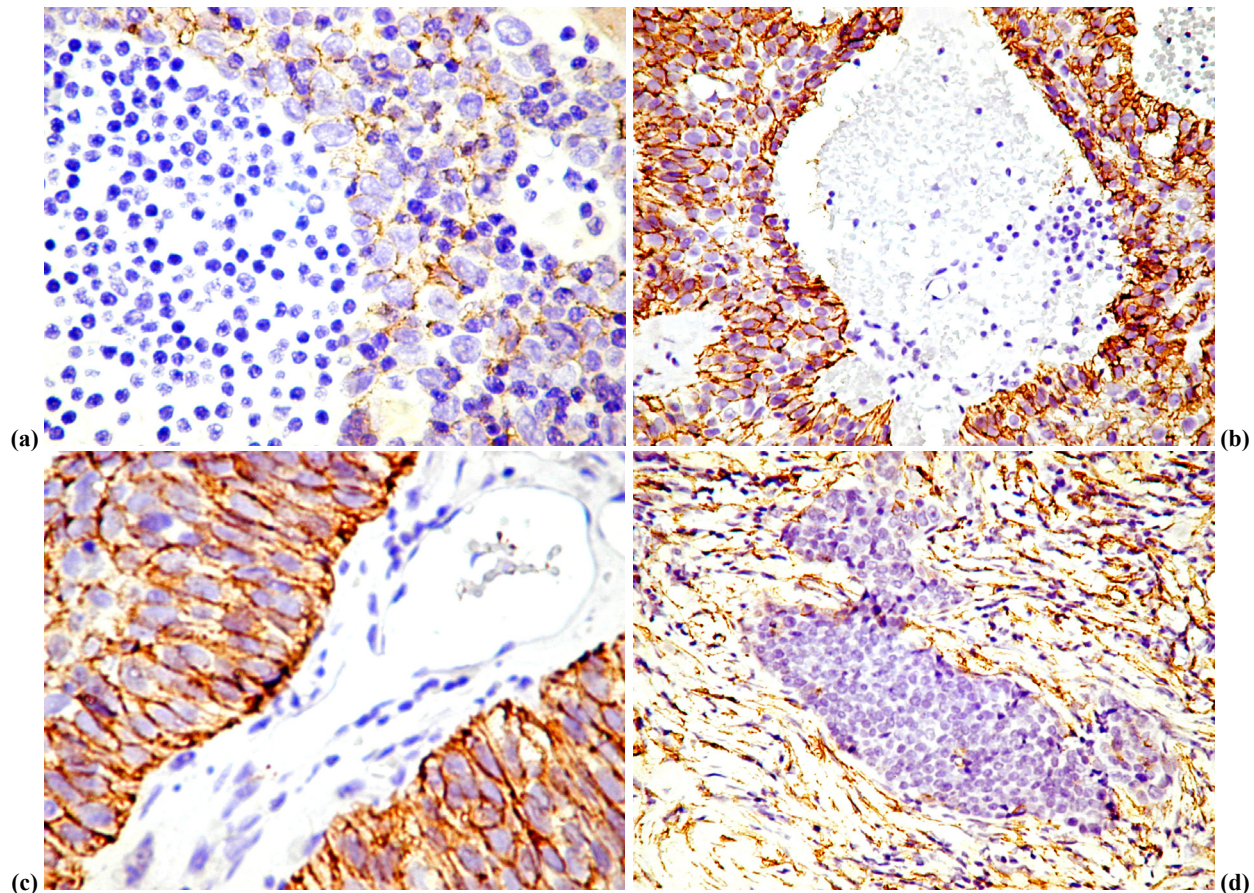


Figure 3 – Weak to moderate reaction in thymoma type B2 (a, $\times 400$). Strong reaction in all neoplastic cells, enhanced around the perivascular space (b, $\times 200$). Increased expression of D2-40 in tumor cells at the invasive front (c, $\times 400$). Positive myofibroblasts in the stroma associated with negative tumor cells (d, $\times 200$). Anti-D2-40 staining.

Discussion

Many works were performed in the last decades to clarify the basic mechanisms of thymus development and behavior in the postnatal life. It was shown that thymus involution is not necessarily a linear process and transient involution may occur even before puberty during various physiological and pathological conditions [20]. Recent investigations showed that the thymus retains restorative capacity even in the adults and the complete regeneration is achieved by the activation of thymic epithelial cell progenitors under the influence of

We found positive reaction in one case with thymoma type A, one type AB, four type B1, four type B2, six type B3, and two with thymic carcinoma. The intensity of reaction and the percentage of positive tumor cells were not related to the type of thymoma. In the same tumor entity (e.g. like in thymoma type B2, Figure 3, a and b) the reaction was weak and heterogeneous, or strong labeling almost all tumor cells. Moreover, the intensity of reaction was stronger in neoplastic cells decorating the border of the perivascular spaces (Figure 3b). The same pattern was found at the interface between the tumor and stroma (Figure 3c). A particular aspect was found in one case of thymoma type B3 characterized by a strong desmoplastic reaction of the stroma. The myofibroblasts were intensely stained, but no reaction was found in tumor cells (Figure 3d).

some transcription factors [21]. Although some molecular events during thymus development are nowadays largely accepted based on experimental models, few data are available about the human thymus and many steps are not characterized at protein level. Some growth factors could be involved in thymus organogenesis and growth of thymomas, like vascular endothelial growth factor [22], epidermal growth factor [23], or platelet-derived growth factor [24]. Until now, the functional significance of these growth factors in the normal thymus and thymoma remains elusive. Based on previous findings on the role of podoplanin in cellular proliferation

and migration, we investigated the immunohistochemical expression of D2-40 in the normal thymus and thymoma.

D2-40 recognizes the formalin-insensitive epitope of podoplanin and it is unanimously recognized as a good marker of the lymphatic endothelium. Recent data have shown that the functions of podoplanin are more complex, and its expression was demonstrated in a large variety of normal and neoplastic cells. In the human fetal and postnatal human thymus, we found that the expression of D2-40 is restricted to the subcapsular and cortico-medullary epithelial cells, which are more likely to proliferate during thymus development. This is also supported by the lack of expression of this marker in the thymus involution, as found in the present study.

Podoplanin belongs to the family of type-1 transmembrane sialomucin-like glycoproteins. A C-type lectin-like receptor-2 (CLEC-2) was identified as an endogenous receptor of podoplanin on platelets [25]. By CLEC-2-Fc deletion mutants, it was demonstrated inhibition of podoplanin-induced platelet aggregation, and this indicates that CLEC-2 is a physiological ligand of podoplanin [26]. Recombinant CLEC-2 has inhibited platelet aggregation induced by podoplanin-expressing tumor cells and LECs [25]. These findings suggest that CLEC-2 is a physiological target protein of podoplanin and the interaction between podoplanin and CLEC-2 may regulate tumor invasion and metastasis. This molecular mechanism could explain our results in patients with thymoma, because we found a strong correlation between D2-40 expression and invasion. Based on these findings, we can speculate that D2-40 is an individual marker of prognosis, and moreover, it can be evaluated as a potential target for therapy in selected cases.

The molecular phenotype of cells in the invasion front is frequently different from that of cells in the tumor core [27]. It was reported that podoplanin-expressing cells are found at the invasion front in more than 80% human squamous cell carcinomas [28]. This is in accord with our findings that showed a stronger expression in neoplastic cells bordering perivascular spaces and the front of invasion. The involvement of podoplanin in tumor invasion is poorly understood, but an explanation might come from the ability of podoplanin to remodel the actin cytoskeleton of tumor cells, contributing to their increased motility [29].

To the best of our knowledge, this is the first report on the expression of D2-40 in thymoma and this event is associated with invasion and poor prognosis. Previous reports have shown positive reaction for podoplanin in germ cell tumors of the thymus, but without any mention regarding epithelial cells of the remaining thymus. In respect to the high percent of D2-40-positive thymomas found in the present study, we may conclude that this marker is not useful to discriminate between thymoma and germ cell tumors of the thymus.

✉ Conclusions

In summary, in the present work we have shown that D2-40 is expressed early during the thymus organogenesis, its expression is retained in some specific

epithelial cells of the normal thymus in the postnatal life and significantly decreases during physiological involution. The expression of D2-40 in thymoma does not correlate with the histological type but correlates with stage of the tumor. Based on these data, it is suggested that expression of D2-40 in thymoma can be used as an individual prognostic factor.

Acknowledgements

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