Abstract. The thymus plays an essential role in the maturing of progenitor cells to functional T cells. Recent studies suggest that the Hedgehog (Hh) signaling pathway contributes to this differentiation process. However, there is limited information concerning the expression of Hh pathway-related proteins (Hh proteins) in the human thymus. The staining of Hh proteins in the thymic epithelium of 26 surgically resected thymoma tissues was examined by immunohistochemistry. The staining of sonic Hh (Shh) correlated relatively well with the World Health Organization histological classification of thymoma. The higher the grade, the fainter the staining. However, no significant difference in Shh staining was found between normal and neoplastic epithelia. Interestingly, Gli1 staining in thymomas was significantly greater than that in normal thymus (p<0.0001). Thus, some members of the Hh signaling pathway may contribute to the development of thymoma.

The thymus is the central lymphoid organ of the immune system in which T cells develop during fetal life; it provides the optimal microenvironment for the maturation of functional T cells (1,2). The basic organization of the thymus involves numerous lobes comprised of an inner central medulla surrounded by an outer cortex. T cell progenitors arrive at the thymus via corticomedullary blood vessels and accumulate in the subcapsula, which is the most external thymic compartment (3). T cell progenitors arriving at the thymus are called thymocytes. In the human thymus, CD4–CD8– double-negative (DN) thymocytes differentiate into mature functional CD4+ or CD8+ T cells through a complex network of signals between thymocytes and thymic stromal cells such as thymic epithelial cells (1, 4-6).

The Hedgehog signaling pathway (Hh pathway) is crucial for the regulation of cell fate and patterning during the development of many organs (7). This pathway consists of several Hh pathway-related proteins (Hh proteins) such as the 12-pass transmembrane protein Patched (Ptch), the 7-pass transmembrane protein Smoothened (Smo) and the 5-zinc finger transcription factor Gli. Binding of Hh ligands such as sonic Hh (Shh) to Ptch induces nuclear translocation of Gli1 and transcription of target genes. Gli1 is also a target gene and, therefore, the expression of Gli1 is considered to reflect Hh pathway activation (8).

Involvement of the Hh pathway in thymocyte differentiation has been described in mice (9-11). In brief, Shh is produced by thymic epithelial cells, while its receptor, Ptc, is expressed predominantly by immature DN thymocytes. Addition of anti-Shh antibody to fetal thymus organ cultures accelerates the progression of DN cells toward the double-positive (DP) stage, and treatment with high doses of exogenous Shh arrests thymocyte differentiation at the DN stage. Analysis of Shh−/− embryos has shown that Shh is important for the regulation of thymic cellularity, as well as for the development of DN thymocytes (11). Recent studies have also indicated the participation of the Hh pathway in T cell maturation in the human thymus (12, 13). The Hh pathway is active during early human thymocyte development, and Shh may function in the maintenance of the intrathymic progenitor cell population, promoting its survival and inhibiting IL-7-mediated proliferation and differentiation. To our knowledge, studies of the Hh pathway in the human thymus are limited to those in which the thymus was obtained from children undergoing corrective cardiovascular surgery. There are no data concerning the expression of Hh proteins in the adult

Abbreviations: Hh, Hedgehog; Shh, sonic Hedgehog; Ptc, Patched; Smo, Smoothened; DN, double-negative; DP, double-positive; IL-7, interleukin-7; WHO, World Health Organization.

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human thymus. To study the expression of Hh proteins in the adult human thymus, surgically resected thymoma specimens were analyzed by immunohistochemistry. Also, the relationship between staining for Hh proteins and the World Health Organization (WHO) histological classification of thymoma was analyzed.

Materials and Methods

Clinical samples and staging. Twenty-six patients with primary thymoma underwent resection at the Department of Surgery and Oncology, Kyushu University (Fukuoka, Japan), during the period 1994 through 2003. Informed consent was provided by each patient before surgical treatment. Surgical specimens were fixed in 10% formalin, embedded in paraffin and examined histopathologically. Thymic epithelial tumors were classified according to the WHO criteria (14). The WHO histological classification system is summarized in Table I.

Immunohistochemistry. Single antibody labelling was performed, as described previously (15), but with the following protocol modifications: endogenous peroxidase activity was blocked by 3% 

H$_2$O$_2$ in methanol for 30 minutes at room temperature. Antigen retrieval was performed by boiling sections in 0.01 mol/L sodium citrate (pH 6.0) for 5 minutes. All primary antibody incubations were overnight at 4°C. The primary antibodies used were all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) as follows: Shh (N-19, sc-1194); Ptc (H-267, sc-9016); Smo (H-300, sc-13943); and Gli1 (N-16, sc-6153) (1:250). Secondary antibodies (Shh and Gli1, rabbit anti-goat immunoglobulin; Ptc and Smo, goat anti-rabbit immunoglobulin; Nichirei Co., Ltd., Tokyo, Japan) were applied for 1 hour at room temperature. Immunolabelling was visualized by development of brown pigmentation via a standard 3,3’-diaminobenzidine protocol. The slides were lightly counterstained with hematoxylin.

Statistical analysis. All statistical analyses were performed with SAS Statistical Software (Release 6.12; SAS Institute, Inc., Cary, NC, USA) on a UNIX workstation. The correlations between Hh protein staining and clinicopathological factors, listed in Tables II and III, were analyzed by Fisher’s exact probability test. P<0.05 was considered significant.

Results

Staining of Hh proteins in normal thymic epithelium. Regions of normal thymic epithelium were identified in 20 of the 26 specimens. The expressions of the functional components Shh, Ptc, Smo and Gli1 of the Hh pathway were determined by immunohistochemistry, repeated on at least 2 different days. Shh staining was clearly identified in 16 of the 20 specimens (80%) (Table III and Figure 1). However, only a few specimens showed detectable staining for Ptc, Smo or Gli1.

Staining of Hh proteins in thymoma. All 26 thymic epithelial tumors (thymomas) were classified according to the WHO histological classification system (14) (Table I) and included 2 type A (7.7%), 11 type AB (42.3%), 8 type B1 (30.8%), 4 type B2 (15.4%) and 1 type C (3.8%) tumor. Sex, mean age and association with myasthenia gravis are denoted in relation to the WHO histological classification system in Table II. Shh staining was found in 2 of the 2 type A specimens (100.0%), 7 of the 11 type AB specimens (63.6%), 5 of the 8 type B1 specimens (62.5%), 1 of the 4 type B2 specimens (25.0%) and 0 of the 1 type C specimen (0.0%) (Table III and Figure 1). Shh staining of types B2 and C was relatively faint compared with that in types A, AB and B1. Staining for Ptc was detected in 2 of the 2 type A specimens (100.0%) and 1 of the 11 type AB specimens (9.1%). Staining for Smo was detected in 1 of the 2 type A specimens (50.0%) and 1 of the 8 type B1 specimens (12.5%). Gli1 staining was detected in 2 of the 2 type A specimens (100.0%), 4 of the 11 type AB specimens (36.4%), 6 of the 8 type B1 specimens (75.0%), 1 of the 4 type B2 specimens (25.0%) and 1 of the 1 type C specimen (100.0%) (Table III and Figure 2). Gli1 staining in thymomas was significantly greater than that in normal thymic epithelium (p<0.0001).
One of the 2 type A specimens (50.0%), 9 of the 11 type AB specimens (81.8%), 6 of the 8 type B1 specimens (75.0%), 3 of the 4 type B2 specimens (75.0%) and 1 of the type C specimen (100.0%) were associated with myasthenia gravis. There was no correlation between WHO classifications and myasthenia gravis and no significant relationship was found between myasthenia gravis and Hh protein staining.

Although the thymic epithelium may play a crucial role in intrathymic T cell expansion and/or differentiation, no significant correlation was found between Shh staining in thymomas and the number of tumor-infiltrating lymphocytes. Unfortunately, we could not determine by immunohistochemistry whether these infiltrating lymphocytes (thymocytes) express Hh proteins.

### Discussion

We examined immunohistochemical staining for Hh proteins in surgically resected human thymoma specimens. With the use of these specimens, we were able to analyze the expression of Hh proteins in normal adult thymic epithelium. Our data suggest that Shh expression in the adult human thymic epithelium is similar to that in children aged 1 month to 3 years (12, 13). Thus, adult thymic epithelium may also function in the intrathymic differentiation of thymocytes. Neoplastic thymic epithelium of types A, AB and B1 may also be functional. The function of the neoplastic epithelium in types B2 and C may be at least partially impaired; Shh staining in these types was faint. However, Shh staining of the neoplastic thymic epithelium was not associated with the number of thymoma-infiltrating lymphocytes. Our data suggest that Shh, secreted by the neoplastic epithelium, is not significantly involved in lymphocyte accumulation.

The prognosis of types B2, B3 and C is generally considered poor compared to that of types A, AB and B1 (16, 17). We found no correlation between WHO classifications and myasthenia gravis. In addition, no significant correlation was found between Shh staining and myasthenia gravis. However, the Shh staining in types B2 and C was relatively faint compared to that in the other types. Although types B2 and C comprised very small populations in this study, our data suggest that the expression status of Shh may be a prognostic factor for thymomas.

Recent data have shown an association between Hh pathway activation and development of human tumors (18-20). Interestingly, Gli1 staining in thymomas was significantly greater than that in the normal thymic epithelium \(* p<0.0001\). Although our data suggest a contribution of the Hh pathway activation to the
Figure 1. Immunohistochemical staining of Shh in normal thymus and thymoma (x200). Representative specimens are shown. Shh staining was found in normal thymic epithelium. In type A, type AB, type B1 and type B2 thymomas, epithelial tumor cells showed detectable levels of Shh staining. No Shh staining was detected in type C thymoma.

Figure 2. Immunohistochemical staining of Gli1 in normal thymus and thymoma (x200). Representative specimens are shown. No Gli1 staining was found in normal thymic epithelium. Many thymomas, including thymic carcinoma (type C), showed detectable levels of Gli1 staining.
development of thymomas, no correlation was found between Gli1 staining and WHO histological classifications.

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