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Expression of claudins relates to tumour aggressivity, location and recurrence in ependymomas

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Summary. The aim of our study was to assess the nature and importance of claudin expression in grade I-III ependymomas. The expression of claudins 2-5, 7, 10, TWIST, and ZEB1 were investigated in a series of 61 ependymomas using immunohistochemistry. All the claudins were expressed in ependymomas, except for CLDN4. CLDN5 positive tumours were associated with higher grade (p=0.049), whereas CLDN10 was lower in higher grade tumours (p=0.039). CLDN5 and CLDN3 were overexpressed in ependymomas of cerebral location (p=0.036, p=0.007, respectively). CLDN5 positive tumours showed more nuclear atypia, endothelial proliferation, mitosis, and hypercellularity (p=0.007, p=0.018, p=0.041, p=0.010, respectively). CLDN5 positivity correlated to higher proliferation (p=0.015). CLDN7 was more often positive in primary tumours (p=0.041). Positive ZEB1 expression was associated with CLDN2 negativity (p=0.031). TWIST-negative tumours were more often also CLDN5 and 10 negative (p=0.013, p=0.017, respectively). CLDN5 was related to more aggressive tumours compared to CLDN2 and 10, which tended to display a better degree of differentiation and a better prognosis. CLDN2 and CLDN5 were expressed commonly in ependymomas, while the parental ependymal cells in the central nervous system were usually negative. Evidently, claudins influence growth and differentiation in ependymomas.

Key words: Claudins, Ependymomas, TWIST, ZEB1

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Introduction

Ependymomas, which comprise approximately 4% of the glial tumours, arise from ependymal cells which are neuroepithelial cells or their progenitor/stem cells (Louis et al., 2007). The tumours are most frequently encountered in young children (Reni et al., 2007; Zacharoulis et al., 2008), whereas the age distribution peak among adults is broader. According to the classification scheme by the World Health Organization (WHO), ependymomas are grade II or grade III (anaplastic) tumours, grade I being designated to rare subgroups of myxopapillary ependymomas (typically present at the conus-cauda equina-filum terminale region) and subependymomas. Ependymomas are heterogenous and have genetic differences (Hirose et al., 2001; Rousseau et al., 2003; Taylor et al., 2005). The etiology of ependymoma is mostly unknown but an increased incidence has been demonstrated in patients with neurofibromatosis type 2 (Reni et al., 2007).

Maximal resection of the tumour provides the basis for the treatment of patients with ependymoma, and adjuvant radiotherapy is often used for improving local control (Swanson et al., 2011). The role of chemotherapy in treatment has so far been modest, but the need for potentially toxic radiation could be postponed by chemotherapy in some cases of young patients (Needle et al., 1997). Patient prognosis is generally better than that with oligodendroglial or astrocytic gliomas (Louis et al., 2007). However, ependymomas have a tendency to recur, as approximately 50% of the patients will experience tumour recurrence. Even very late recurrences have been shown to occur (Zacharoulis and Moreno, 2009). Conventional prognostic factors include location of the tumour, patient age, tumour grade and the

extent of surgery (Jeuken et al., 2002; Korshunov et al., 2003; Swanson et al., 2011). Interestingly, the location of the tumour seems to be the most important prognostic factor even when compared to tumour grade (Louis et al. 2007).

Claudins (CLDNs) are a family of tight junctional proteins which regulate the paracellular permeability of cellular layers and influence cellular polarity and orientation (Turksen and Troy, 2000). They also serve as a fence, separating different cell membrane compartments from each other (Turksen and Troy, 2000). Claudins have been shown to be expressed in epithelial, endothelial and mesothelial cells, as well as in their corresponding tumours (Soini, 2005; Soini et al., 2006a; Ouban and Ahmed, 2010). To date, twenty-seven different claudins have been identified (Mineta et al., 2011). Claudins are essential tight junction proteins that form the backbone of blood-brain-barrier (Louis et al., 2007). The expression of claudins appears to vary between tissues and between different sites in individual organs (Turksen and Troy, 2000; Ouban and Ahmed, 2010). In kidney tubuli, for instance, changes in the expression profile have been associated with differences in the permeability of individual tubular segments (Kiuchi-Saishin et al., 2002). Similar fluctuation in the expression of individual claudins has been demonstrated between mucosal epithelial cells of the gut (Rahner et al., 2001). Considering the central nervous system, claudins 3 and 5 have been linked to the sealing function of the blood-brain-barrier, and expression of claudins 1, 2 and 11 has been demonstrated in the choroid plexus epithelium, i.e. the barrier for cerebrospinal fluid (Wolburg et al., 2001, 2003; Nitta et al., 2003).

In cancer, deregulation of claudins and dysfunction of these proteins cause changes in the microenvironment of the cells and loss of cell-cell adhesion, leading to disturbances in cellular differentiation, uncontrolled cell proliferation, loss of cohesion and increased invasiveness (Oliveira and Morgado-Diaz, 2007). The pathway involves changes in markers associated with epithelial-mesenchymal transition (EMT). The expression of claudins appears to vary in a tumour specific manner. Moreover, histological subtypes of tumours may also show distinctive expression patterns

Table 1. Characteristics of ependymomas of the brain and spinal cord.

	All locations	Spine	Cerebellum	Cerebrum	p-value
Primary tumours					
Age (mean, yrs)	30.91±20.2	35.56±16.07	23±24.67	31.71±20.22	0.250*
Sex					
0.814 **					
Females	19	7	5	7	
Males	25	11	7	7	
Alive/dead	44/17	18/0	8/4	8/6	0.028***
All tumours					
Ki-67 (MIB-1), median (mean±SD)	0.78 (3.08±5.20)	0.49 (1.04±1.21)	0.41 (4.30±7.06)	2.25 (4.30±5.49)	0.425****
CLDN 2	42%	55%	27%	40%	0.235**
Positive	24/57	12/22	4/15	8/20	
Normal ependyma	*negative*	*negative*	*negative*		
CLDN 3	23%	14%	6%	47%	0.007**
Positive	13/57	3/22	1/16	9/19	
Normal ependyma	*negative*	*positive*	*positive*		
CLDN 4	0%	0%	0%	0%	n.s.
Positive	0	0	0	0	
Normal ependyma	*Negative*	*Negative*	*Negative*		
CLDN 5	26%	18%	13%	47%	0.036**
Positive	15/57	4/22	2/16	9/19	
Normal ependyma	*Negative*	*Positive*	*Negative*		
CLDN 7	81%	87%	87%	70%	0.298**
Positive	47/58	20/23	13/15	14/20	
Normal ependyma	*Negative*	*Positive*	*Positive*		
CLDN 10	11%	14%	6%	11%	0.765**
Positive	6/57	3/22	1/16	2/19	
Normal ependyma	*Negative*	*Negative*	*Negative*		

For comparison claudin expression of different levels of normal ependyma in autopsy material is also shown (italics). *: One-Way variance test; **: Chi-square test; ***: Log rank test; ****: Kruskall-Wallis test.

(Soini, 2005; Hewitt et al., 2006). As examples, in lung cancer, CLDN3 and CLD5 are expressed less frequently in squamous carcinoma than in adenocarcinoma (Paschoud et al., 2007; Moldvay et al., 2007). In gastric cancer, increased expression of claudins characterizes the intestinal rather than the diffuse carcinoma type (Soini et al., 2006b). However, relatively little is known about claudin expression in tumours of the central nervous system. Meningiomas have been shown to express claudins 1 and 11 (Soini et al., 2010), and down-regulation of claudins 1 and 5 appears to characterize the microvasculature of glioblastomas (Liebner et al., 2000).

In this study, we investigated the expression of claudins 2-5, 7 and 10 by immunohistochemistry in a series of 61 ependymomas. The results were compared with tumour histopathology, clinical data and proliferation index. Additional interest was paid to two transcription factors, TWIST and ZEB1, respectively, which participate in EMT and might thus be relevant in claudin regulation.

Materials and methods

A total of 61 tumours of ependymal origin were collected for the study (44 primary tumours, a re-occurring tumour in four cases, and 13 patients with recurrence only). All 57 patients underwent neurosurgical operation with the intention of gross total extirpation of the tumour mass between January 1979 and May 1999 at Tampere University Hospital, Kuopio University Hospital and Turku University Hospital, Finland. The median age of the 44 patients with primary tumours was 33 years (mean 30.9±20.2, range 3.5 months to 67 years) at the time of diagnosis (Table 1). Twenty-five of them were male and 19 were female. The median follow-up period was 6.4 years. The location of the primary tumours varied as follows: the cerebrum (32%), the cerebellum (27%), and the spinal cord (41%). Postoperative adjuvant therapy did not follow a uniform practice, as the clinical data showed application of radiotherapy in several and chemotherapy in only a few cases.

All the tumour samples were fixed in 4% phosphate buffered formaldehyde and processed into paraffin blocks. The sections were stained with hematoxylin and eosin (HE). An experienced neuropathologist evaluated the tumour samples and determined the histopathological type and grade according to the criteria presented by the WHO (Louis et al., 2007) (Table 2). The neuropathologist pinpointed the most representative tumour area, from which a sample was chosen for multitissue microarray processing. The microarray blocks were constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA). The sample diameter of the tissue core was 600 μm in the microarray block.

The whole set of HE-slides used in the primary histological diagnosis was available in cases of Tampere University Hospital (N=23). The tissue microarrays comprised the total tumour material (N=61). In these 23 ependymomas the presence or absence of the following histological features were analyzed: atypia, endothelial proliferation, necrosis, mitosis, hypercellularity, pseudorosettes, true rosettes, papillarity, calcification, and myxoid change.

Immunohistochemical staining for claudins

The primary antibodies for the detection of claudins 2-5 and 7 and 10 in formalin-fixed paraffin-embedded tissues were purchased from Zymed Laboratories Inc (South San Francisco, CA). The antibodies included polyclonal rabbit anti-claudin 2 (clone JAY.8), polyclonal rabbit anti-claudin 3 (clone Z23.JM), monoclonal mouse anti-claudin 4 (clone 3E2C1), monoclonal mouse anti-claudin 5 (clone 4C3C2), polyclonal rabbit anti-claudin 7 (clone ZMD.241) and polyclonal anti-rabbit claudin 10 (Ca N:o 38-8400). The microarray sections were first heated in a microwave oven in 10 mM citrate buffer (pH 6.0) for 10 minutes. After a 60-minute incubation with the primary antibody (dilution 1:50 for anti-claudin 1, 3, 4, 5 and 7, 1:100 for

Table 2. Characteristics of the ependymal tumours by grade.

	Grade I	Grade II	Grade III	p-value
primary	9	28	7	
recurrent	2	11	4	0.702
Primary tumours				
Age (<19/≥19yrs)	2/8	9/19	5/1	0.029
Sex (female/male)	2/8	14/14	3/3	0.242
Survival (alive/dead)	8/1	20/8	6/1	0.572*
All tumours				
Location				0.001
spinal	10	12	1	
cerebellum	0	14	4	
cerebrum	1	13	6	
CLDN 2				
negative	5	22	6	0.763
positive	5	16	3	
CLDN 3				0.912
negative	7	28	9	
positive	2	9	2	
CLDN 5				0.049
negative	8	29	5	
positive	1	8	6	
CLDN 7				0.613
negative	2	6	3	
positive	9	31	7	
CLDN 10				0.039
negative	6	34	11	
positive	3	3	0	

All grade I tumours were myxopapillary ependymomas. Chi-square and * log rank tests used.

claudin 10), a biotinylated secondary anti-rabbit or anti-mouse antibody and Histostain-SP kit (Zymed Laboratoris Inc) were used. The bound antibodies were demonstrated with diaminobenzidine as a chromogen. The sections were then lightly counterstained with haematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Positive controls included non-neoplastic kidney, breast, skin and liver samples. Non-immune rabbit or mouse serum as substitutes for the primary antibodies were used as the negative control.

Two observers (YS, HH) analyzed the staining results, and a consensus meeting was held in the case of a disagreement. Membrane bound immunoreactivity was considered significant, although cytoplasmic expression was also detected to be present. The immunoexpression of claudins was assessed as follows; negative = <5% of cells positive, weak = 5-25 % of cells positive, moderate = 25-75 % of cells positive, strong = 75-100 % of cells positive. Due to the sample size, the tumours were recorded as CLDN-negative or CLDN-positive (from weak to strong staining) for statistical analyses.

To evaluate claudin staining in non-neoplastic formalin-fixed and paraffin-embedded central nervous system tissue, samples of normal ependyma were obtained from five autopsies performed within 36 hours after a sudden death. Representative samples of lateral ventricle (cerebrum), IVth ventricle (cerebellum) and central canal of spinal cord were collected according to a standard neuropathological autopsy protocol.

Immunostaining for TWIST and ZEB1

The microarray sections were incubated overnight at 4°C with the mouse monoclonal TWIST (ab50887, Abcam, Cambridge, UK) and ZEB1 (clone 416A7H10, GenWay, San Diego, CA, USA) antibodies at a 1:500 dilution for both. The slides were then stained using a standard avidin-biotin-enhanced immunoperoxidase technique (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine tetrahydrocloride (DAP, in phosphate-buffered saline) (Sigma, St. Louis, MO, USA) was used as chromogen. The sections were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted with Depex (BDH, Poole, UK). Ovarian tumour tissue, known to be positive for TWIST and ZEB1 expression, was used as a positive control. Nuclear immuno-reactivity for TWIST and ZEB1 was considered significant. Tumours with staining with over 5% positivity were recorded TWIST/ZEB1-positive.

Immunostaining for Ki67

The monoclonal antibody MIB-1 (Ki-67 antigen, dilution 1:40, Immunotech, S.A. Marseille, France) was used for cell proliferation analyses. Counterstaining was done using methyl green. A computer-assisted image analysis system (CAS-200 TM Software) was utilized for the evaluation of the percentage of MIB-1 positive nuclei in tissues, as described earlier (Sallinen et al., 1994).

Statistical analysis

All statistical analyses were done using SPSS 11.0 for Windows (Chicago, IL). The significance of associations was defined using chi-square test, Mann-Whitney test, and Kruskal-Wallis test. The log rank test and Kaplan-Meier curves were used in the survival analysis. Values ≤0.05 were considered statistically significant.

Results

Claudin expression in ependymomas

Examples of the immunohistochemical expression of claudins in ependymomas are given in Fig. 1. Table 1 shows the expression pattern of claudins in ependymal tumours and normal ependyma. Forty-two percent (N=24) of all ependymomas were positive for CLDN2 (12% strong, 16% moderate, 14% weak), and 23% (N=13) of the tumours were positive for CLDN3 (7% strong, 7% moderate, 9% weak). CLDN4 was not expressed in ependymomas at all. 15 ependymomas were positive for CLDN5; strong CLDN5 expression was found in 16%, moderate expression in 5%, and weak expression in 5% of the tumours. Most (81%, N=47) of the tumours expressed CLDN7 (5% strong, 31% moderate, 45% weak), and 11% (N=6) of the cases were positive for CLDN10 (0% strong, 4% moderate, 7% weak).

Association of claudin expression with clinical and histological parameters

CLDN5 immunopositivity appeared to be associated with high tumour grade (p=0.049, chi-square test), whereas CLDN10 positive ependymomas were often low grade tumours (p=0.039, chi-square test), Table 2. Tumours of the cerebrum were more frequently found to be positive for both CLDN5 and CLDN3 than tumours in the cerebellum or the spinal cord (p=0.036, p=0.007, chi-square test, respectively) Table 1. Added to this, CLDN3 negative tumours were often found negative also for CLDN5 (p=0.010, chi-square test). None of the other claudins had correlations with each other.

CLDN5 immunoreactivity was associated with increased nuclear atypia, endothelial proliferation, mitotic rate and hypercellularity (p=0.007, p=0.018, p=0.041, p=0.010, chi-square test, respectively). CLDN5 positive tumours also showed a higher Ki-67/MIB-1 cell proliferation rate than CLDN5 negative tumours (p=0.015, Mann-Whitney test). The immunoexpression of the other claudins was not associated with histological features or cell proliferation index of ependymomas.

The expression patterns of CLDNs were not associated with patient prognosis. Nevertheless, analyses

indicate that patients with tumours expressing CLDN2 or CLDN10 may have a better outcome than those patients with tumours positive for CLDN 3 and CLDN7 (Fig. 2). None of the patients with CLDN10 positive tumours died during the follow-up. In addition, those patients with CLDN7 negative tumours were alive at the end of the follow-up. CLDN7 was also the only claudin that showed a statistically significant difference in expression between primary and recurrent tumours, as CLDN7 immunoreactivity was more often found in primary than in reoccuring ependymomas (p=0.041, chi-square test). There were no significant associations when CLDNs, TWIST and ZEB1 were compared to different treatment modalities in recurrent ependymomas (p=n.s., chi-square test).

Expression of ZEB1 and TWIST in ependymoma

The immunohistochemical expression of ZEB1 and TWIST in ependymomas is demonstrated in Fig. 3. Tumours positive for ZEB1 were often negative for CLDN2 (p=0.031, chi-square test). Tumours negative for TWIST were often also negative for CLDN5 and CLDN10 (p=0.013, p=0.017, chi-square test, respectively). Added to this, we analysed the association between ZEB1 or TWIST and CLDNs in different ependymoma subgroups dividing the tumours according to their grade. We found that grade II ependymomas negative for TWIST were more often negative for CLDN3 as well (p=0.044, chi-square test). None of the other claudins had such associations. When comparison of claudin, TWIST and ZEB1 was performed separately between grade I ependymomas and grades II-III were combined, only CLDN10 expression differed significantly (p=0.044, chi-square test). Neither ZEB1 nor TWIST associated with tumour grade or location (p=n.s., chi-square test, respectively), cell proliferation rate (p=n.s., Mann-Whitney test), histological features

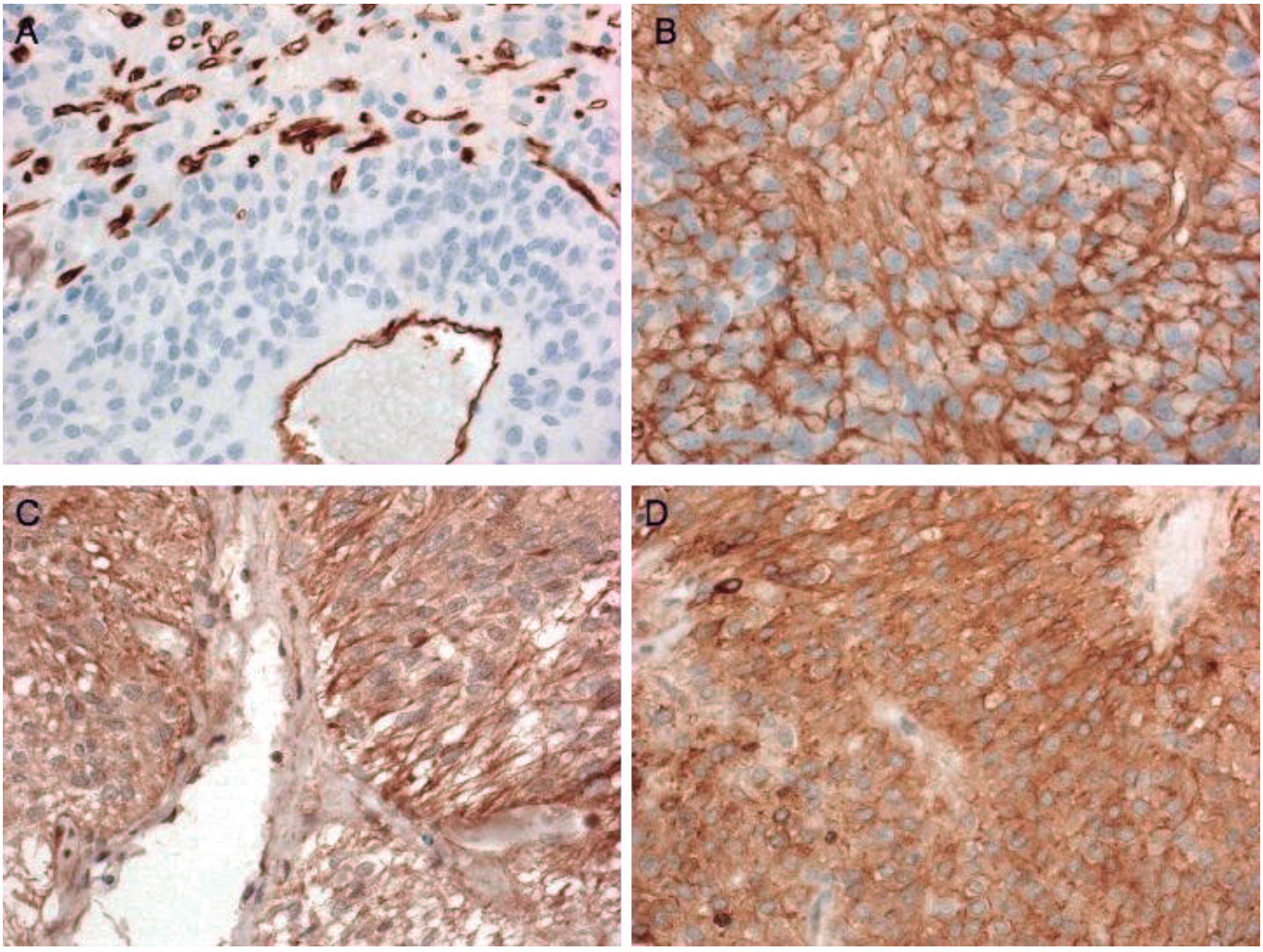

Fig. 1. Examples of expression of claudins (CLDNs) in ependymomas as detected by immunohistochemistry: **A.** Negative expression for CLDN5, **B.** Positive reaction for CLDN5, **C.** CLDN7 positivity, **D.** Positive expression for CLDN10. x 200

(p=n.s., chi-square test), between primary and recurrent tumours (p=n.s., chi-square test) or patient prognosis (p=n.s., log rank test).

Discussion

Claudins are essential tight junction proteins that form the backbone of the blood-brain-barrier (Oliveira and Morgado-Diaz, 2007). The expression of claudins has been shown to be down- or up-regulated in various human tumours (Singh et al., 2010). Decreased expression could indicate the loss of tight junctions during tumourigenetic transformation of cells, and an increase in CLDN expression could be explained by direct oncogenic involvement of claudins in signaling pathways mediated through the scaffolding proteins of tight junctions. Interestingly, the regulation of claudins appears to differ in a tumour specific manner, which underlines the heterogeneity among human neoplasms and offers a novel approach to distinguish tumours by their claudin expression profiles for diagnostic as well as therapeutic purposes (Hewitt et al., 2006).

In our material, claudins were widely expressed in ependymomas. CLDNs seemed to be even more highly expressed than in the previous study, where mRNA expression of eight ependymomas was analysed along with a huge sample set of other tumours (Hewitt et al., 2006). A strong and intensive staining pattern was detected with CLDN5, which is also in line with Hewitt et al. (2006) showing a correspondingly high claudin 5 mRNA expression in ependymomas and other brain tumours. Similarly, we also found strong expression of claudins 2 and 10, but the strongest expression was found for claudin 7, of which the mRNA expression was, however, reported low (Hewitt et al., 2006). Claudins 3 was relatively weakly expressed and no expression was found for claudin 4, a finding also in keeping with the mRNA analysis of Hewitt et al. (2006). Considering CLDN7, the discrepancy could result from a more stable CLDN7 protein expression pattern than that of the other claudins in ependymomas. On the other hand, we had a considerably higher amount of cases in our analysis with data on the tumour location and recurrence.

The normal spinal canal ependyma was immunohistochemically negative with all claudins, ZEB1 and TWIST. However, there was a difference only in CLDN3 and 5 staining between the spinal canal ependymomas and tumours of other locations (Table 1). In addition, when comparison of claudin, TWIST and ZEB1 expression was performed separately between grade I (mostly myxopapillary) ependymomas and combined grades II and III, only CLND10 expression differed significantly. These results show that although ependymomas represent different molecular and morphological subtypes, there are surprisingly many similarities between these tumours of different location and genetic background.

CLDN 3 and 5 were more often found in

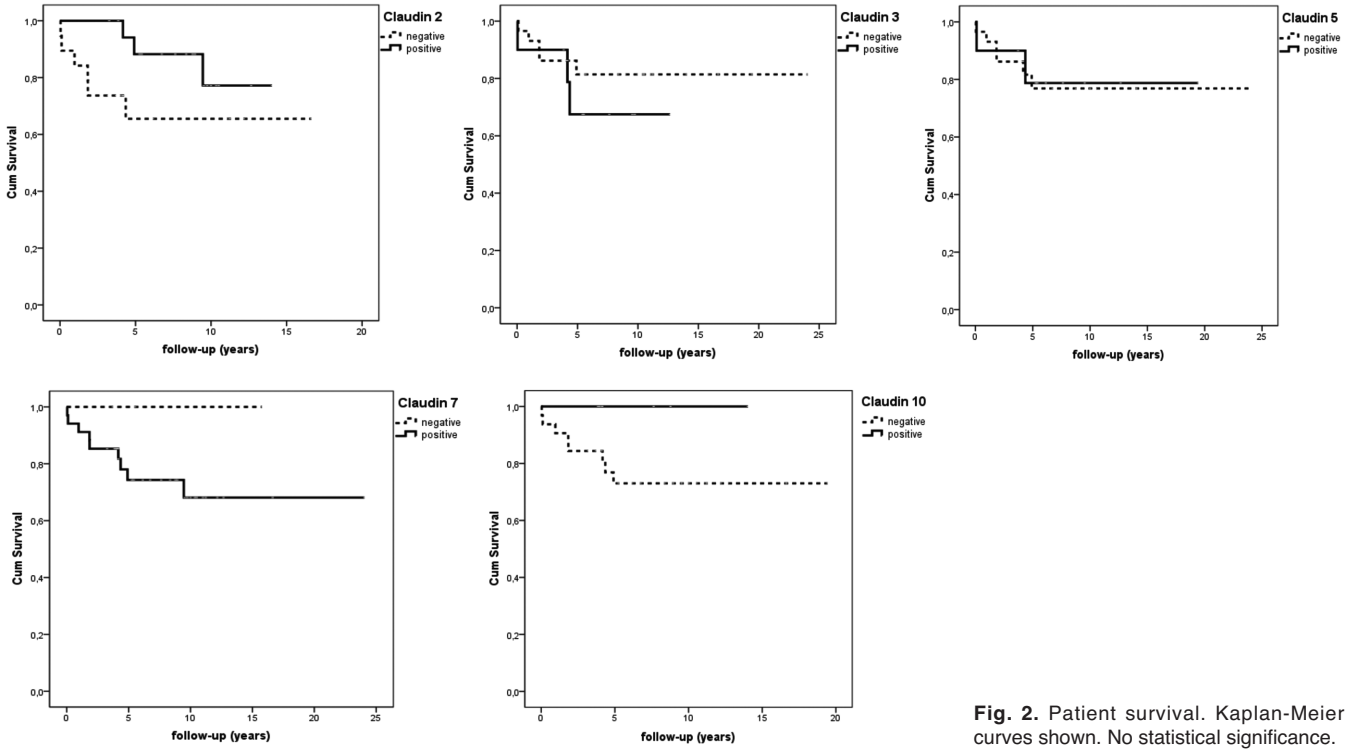

Fig. 2. Patient survival. Kaplan-Meier curves shown. No statistical significance.

ependymomas located in cerebrum than in cerebellum or spinal cord. Claudin 3 was positive especially in ependymomas of cerebrum where the normal ependymal lining was positive as well. On the contrary, claudin 5 was positive in one half of the cerebral ependymomas, but the normal cerebral ependyma was negative for this claudin. Such regional variation in expression may originate from the expression pattern of parental ependymal cells lining the ventricular wall, which may vary similarly to that shown in other tissues (Kiuchi-Saishin, 2002; Rahner et al., 2001). In the study of Mullier et al. (2010), mouse tanyocytes of the hypothalamic region expressed only claudins 1 and 5. On one hand, claudin expression in ependymal cells may be different in humans. On the other hand, neoplastic changes in cells may give rise to abnormal expression of some claudins in ependymomas, like claudin 3.

Claudin 7 was positive in most of the ependymomas of different locations and was also positive also in the normal ependyma of the brain. CLDN 7 was more often seen in primary tumours than in recurrent ones. CLDN 7 is a member of the family, which is abundantly present in epithelial tumours (Soini, 2005). However, depending on tumour type, claudin 7 expression may be over- or underexpressed. Claudin 7 is downregulated in malignant melanoma and head and neck cancer. In breast cancer, an aggressive behaviour has been associated with downregulation of CLDN7 (Krämer et al., 2000; Kominsky et al., 2003). This is reasonable considering the disruption of tight junctions leading to loss of cohesion and invasiveness. Claudin 7 may, however, be upregulated in some other tumours, such as hepatocellular carcinoma, ovarian carcinoma, prostate carcinoma or renal chromophobe carcinoma (Singh et al., 2010). Our results suggest a downregulation of CLDN7 in ependymoma progression, but curiously, all

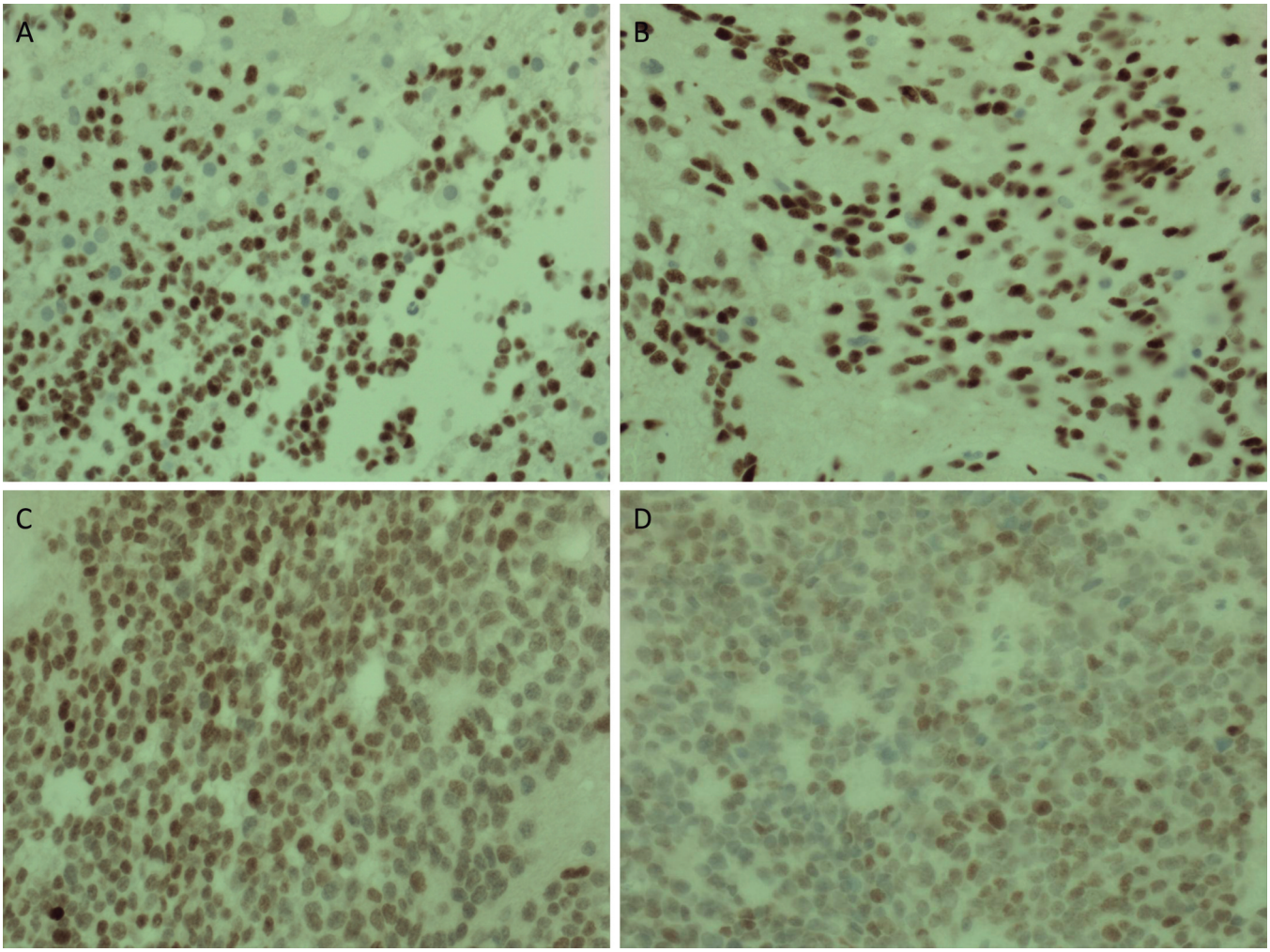

Fig. 3. Examples of expression of TWIST and ZEB1 in ependymomas as detected by immunohistochemistry: Strong nuclear staining was observed for both ZEB1 and TWIST in these cases of ependymomas in the majority of tumour cells. **A.** ZEB1, **B.** ZEB1 **C.** TWIST, **D**. TWIST. x 200

patients with CLDN7 negative tumours survived, in contrast to the CLDN7 positive tumour group.

Claudin 10 was negative in the ependyma of all levels of the central nervous system and was usually negative in the ependymomas studied. CLND10 has been infrequently studied in tumours. Expression has been detected in gallbladder cancer, bile duct cancer and in hepatocellular carcinoma (Németh et al., 2009). CLDN10 was mostly present in intrahepatic bile duct cancer and in hepatocellular carcinoma, where CLND10 mRNA expression was associated with disease recurrency (Cheung et al., 2005; Németh et al., 2009). Elevated CLDN10 mRNA expression levels have also been detected in papillary thyroid carcinomas, while follicular type carcinomas appeared CLDN10 negative (Aldred et al., 2004). Our study showed immunoexpression of claudin 10 in 11 % of cases, and the expression tended to characterize low grade tumours with a favourable prognosis.

Similarly, claudin 2 tended to be expressed in ependymomas with a better prognosis. Claudin 2 is commonly present in epithelial tumours and, similar to claudins 7 and 10, its presence is associated with leakiness of the tight junctions (Soini, 2005; Krause et al., 2008). Claudin 2 was negative in normal ependyma, whereas positive expression was found in 42 % of the tumours, indicating the possible association to tumorigenesis. This finding is comparable to that of epithelial tumours (Soini, 2005). This is in line with previous studies, in which cell lines decreased claudin 2 expression increases cellular motility (Ikari et al., 2011). Furthermore, claudin 2 is increased in chronic colitis and colitis associated dysplasia, and it has been suggested that CLDN2 has a role in early neoplastic transformation of epithelial cells in chronic inflammatory bowel disease (Weber et al., 2008).

Claudin 4 was not expressed at all in either normal or neoplastic ependyma. This is in contrast to epithelial tumours of which the majority express claudin 4 (Soini, 2005; Ouban and Ahmed, 2010). On the other hand, ependymomas seem to express a similar kind of profile in claudin expression to that which is observed in other brain tumours involving a comparatively low expression level of claudins 3 and 4, by which they clearly differ from carcinomas (Hewitt et al., 2006). The low expression of claudin 4 is unfortunate since claudins 3 and 4 contain a binding site for clostridium perfringens cytotoxin (CPE) in their smaller extracellular loop (Gao et al., 2011). Experimental treatment of ovarian carcinoma cells with modified CPE led to destruction of tumour cells and an enhanced sensitivity to chemotherapeutic agents. Interestingly, treatment of epithelial metastasis in mice with CPE led to a reduction in metastatic tumours in mice (Kominsky et al., 2007), and CLND3 and 4 have potential in the targeted therapy of tumours by CPE (Walther et al., 2012).

The expression of claudins is partly regulated by EMT associated transcription factors, such as snail1, slug, ZEB1 or TWIST (Martinez-Estrada et al., 2006). An inverse association between ZEB1 and claudins 1 and 2, and TWIST and claudin 5 has been shown in lung carcinoma (Merikallio et al., 2011). Moreover, the activity of claudins 1, 2, 3, 4 and 7 is regulated by the transcription factors snail1 and slug (Ikenouchi et al., 2003; Martinez-Estrada et al., 2006; Medici et al., 2006). Because of this we tested whether there would be any indication of such regulation in ependymomas by ZEB1 or TWIST. Indeed, ZEB1 was inversely associated with claudin 2 in ependymomas, analogously to lung carcinomas, but claudins 5 and 10 showed a positive correlation with TWIST. Such a discrepancy may be due to the special nature of the ependymal cells and neoplasms, which do not straightforwardly correspond to epithelial cells or tumours.

Ependymomas contained a high frequency of ZEB1 expression compared to e.g. lung carcinomas (Merikallio et al., 2011). This may be due to the importance of ZEB1 in neural development. In mice, activation of ZEB1 mRNA expression is found in cells of the periventricular area (Yen et al., 2001). Thus, ZEB1 might serve other functions in ependymoma, not necessarily related to invasion type features like EMT. TWIST expression was an infrequent finding in ependymomas. In glioblastoma cells, TWIST has been found to be related to an aggressive behaviour and mesenchymal change (Mikheeva et al., 2010). Interestingly, glioblastoma cell lines did not use the cadherin switch, i.e. no changes in the expression of E-cadherin or N-cadherin and TWIST were observed during mesenchymal change (Mikheeva et al., 2010).

The tumour site has been identified as the most important prognostic factor in ependymomas (McLendon et al., 2007), even more important than the histological grade (Grill et al., 2001). Children more commonly have ependymoma in the posterior fossa than adults, where the tumour is known to behave more aggressively and patients have worse prognosis. Although CLDN5 correlated with several features commonly associated with poor prognosis in cancer, such as tumour cell proliferation and histological grade, it did not have prognostic significance in ependymal tumours of our study. This might be due to the fact that CLDN 5 was seldom positive in the ependymomas of posterior fossa, only in 13 % of cerebellar cases, while 47 % of cerebral ependymomas were CLDN 5 positive.

The identification of histopathological or molecular features with prognostic value in ependymal tumours has remained a difficult and controversial issue (Ellison et al. 2011). According to the WHO classification of tumours of the central nervous system (McLendon et al., 2007) even the definition of prognostically reliable histopathological indicators of anaplasia in ependymal tumours has not been completely resolved. The same difficulty in the prognostic evaluation of ependymomas is shown in the analysis of molecular features of the present study. However, the limited size of our tumour series might be one reason for this.

In the series of 61 ependymomas we found

differences in the expression of claudins associated with the location of the tumour, as well as between primary and recurrent tumours and between normal ependyma and ependymomas. CLDNs 3 and 5 were more often found in the cerebral ependymal tumours than in other sites. Added to this, primary tumours showed a more intense CLDN7 staining pattern compared with recurrent ones. CLDN5 was related to more aggressive ependymomas when compared to CLDN2 and 10, which tended to be present in tumours of a better degree of differentiation and prognosis. Interestingly, claudin 2 and claudin 5 were expressed commonly in ependymomas while the parental ependymal cells lining the ventricular wall were usually negative. Thus, the results suggest that claudins may have a role in the pathogenesis of ependymal tumours, i.e. claudin 2 more in the tumorigenesis of lower grade ependymomas and claudin 5 in the neoplastic development to higher grades. To assess the prognostic value the expression of claudins should be studied in a larger cohort.

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