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Notch receptors in human choroid plexus tumors

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Summary. Notch signaling plays a role in development and formation of the normal choroid plexus (nCP), and in formation of various tumors in humans. Activation of Notch3 has been reported to promote tumor growth in invasive gliomas and to initiate formation of choroid plexus tumors (CPT) in mice.

We investigated the expression of all currently known Notch receptors (Notch 1-4) in 55 samples of nCP and 88 CPT, including 61 choroid plexus papillomas (CPP), 22 atypical CPP and 5 choroid plexus carcinomas by immunohistochemistry. Notch expression was semiquantitatively evaluated separately for membranous/cytoplasmic and for nuclear staining. In addition, we examined Her2 expression (EGFR2, Her2/neu, ErbB2, CD340) because of its functional link to Notch signaling.

All samples were negative for Notch3. Membranous/cytoplasmic expression of Notch1 ($p<0.0001$) and Notch4 ($p=0.046$) was significantly higher, whereas Notch2 expression was significantly lower ($p<0.0001$) in nCP compared to CPT. Nuclear expression of Notch1, -2 and -4 was significantly higher in CPT compared to nCP ($p<0.0001$ each). Expression of Notch2 and Notch4 showed a shift from a prevailing membranous/cytoplasmic expression in nCP to a predominant nuclear expression in CPT. Her2 was weakly expressed in 42/84 CPT but only in 2/53 nCP ($p=0.0001$) and positively correlated with nuclear expression of Notch1, -2 and 4 in CPT.

In summary, a shift between membranous/cytoplasmic (non-canonical signaling pathway) and nuclear expression (canonical signaling pathway) of Notch1, -2 and -4 and upregulation of Her2 indicate neoplastic transformation in human CP and may reveal new therapeutic approaches.

Key words: Notch receptors, Her2, Chroid plexus epithelium, Choroid plexus tumor

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Introduction

Notch signaling is an evolutionarily conserved, intercellular signaling mechanism that plays myriad roles during vertebrate development and oncogenesis (Callahan and Egan, 2004; Gridley, 2010). In mammals, so far four Notch receptors have been described, which are labeled Notch1-4 (Gridley, 2010).

Notch family receptors are transmembrane proteins at the cell surface consisting of a large extracellular domain and a membrane-tethered intracellular domain. After proteolytic cleavage of the Notch intracellular domain (NICD) by a gamma-secretase complex the cleaved fragment translocates to the nucleus (so called canonical signaling pathway). Once in the nucleus, NICD binds to CBF1 (also known as CSL and RBPJ) and converts CBF1 from a transcriptional repressor to an activator (Mumm and Kopan, 2000; Leong and Karsan, 2006).

Cellular functions and microenvironmental cues associated with tumorigenesis which are modulated by Notch signaling include proliferation, apoptosis, adhesion, epithelial-to-mesenchymal transition, tumor angiogenesis and metastasis (reviewed by Leong and Karsan, 2006; Garcia and Kandel, 2012). Thereby, Notch signaling can act as an inhibitor of apoptosis (Perumalsamy et al., 2009) or promote tumor cell proliferation and angiogenesis (Leong and Karsan, 2006). It is already known that Notch signaling can promote tumorigenesis and tumor progression of hematologic and solid neoplasms, such as carcinomas originating from various visceral organs (Callahan and Egan, 2004; Gao et al., 2008; Bin et al., 2009; Zardawi et al., 2009; Srivastava et al., 2010; Serafin et al., 2011), infantile hemangiomas (Calicchio et al., 2009), hemangioblastomas (Merrill et al., 2011) and amelanoblastomas (Siar et al., 2010) as well as astrocytomas and medulloblastomas (Xu et al., 2009). Notch1 signaling is critical for survival and proliferation of human glioma cell lines (Purow et al., 2005) and in human ependymomas (Puget et al., 2009; Milde et al., 2012). In mice, Notch3 activation promotes formation of invasive gliomas (Pierfelice et al., 2011) and initiates

choroid plexus tumor formation (Dang et al., 2006).

On the other hand, Notch signaling may also act in a tumor suppressive manner through upregulation of p21 and p27, resulting in cell cycle arrest in murine and human cell lines from hematologic and epithelial neoplasms (for review see ref. Leong and Karsan, 2006).

Data on the expression of Notch receptor family members in nCP are rare. In rodents nCP has been reported to express Notch2 and to lack Notch1 and Notch3 at the RNA level (Weinmaster et al., 1992; Higuchi et al., 1995; Lindsell et al., 1996; Irvin et al., 2001). So far, to our best knowledge, there is no data available on Notch expression in CPT.

There is growing information on the crosstalk between Notch receptor family members and Her2 signaling in tumorigenesis. Her2-Notch3 crosstalk is required during early steps in mammary tumorigenesis (Pradeep et al., 2012). Her2 induces Notch1 activity and function in breast cancer cells (Lindsay et al., 2008) and Notch signaling has been proposed to regulate Her2 expression in cancer cells (Magnifico et al., 2009). Her2 overexpression suppresses Notch1 activity in breast tumors (Osipo et al., 2008) and expression of Notch2 correlates with Her2 expression in human breast cancer (Florena et al., 2007). Furthermore, Notch2 appears to counteract the pro-oncogenic effects of Notch1 and Notch4 (O'Neill et al., 2007).

Increasing knowledge of the role of Notch signaling in tumorigenesis and on the crosstalk between Notch and other signaling pathways indicates new opportunities and challenges for Notch-targeted therapies in oncology (Rizzo et al., 2008). For example, Notch2 is a promising new therapeutic target in breast cancer therapy (Florena et al., 2007). Therefore, application of therapeutic monoclonal antibodies targeting individual Notch receptors seems to be superior to pan-Notch inhibitors by reducing or avoiding severe intestinal toxicity (Wu et al., 2010; Sharma et al., 2012).

The aim of this study was to characterize the immunohistochemical expression of Notch receptors (Notch1-4) and Her2 in human samples of nCP and CPT. This data should provide information on Notch and Her2 signaling playing a role in tumor formation and progression of CPT. Furthermore, this study will provide information whether Her2 and/or Notch receptors might be potential therapeutic targets in CPT and which Notch receptors will be the most promising targets for such therapeutic approaches.

Material and methods

Normal and neoplastic choroid plexus samples

We investigated paraffin-embedded tissue samples from normal and neoplastic choroid plexus by immunohistochemistry. Clinicopathological data on investigated cases (nCP and CPT) including age and gender are summarized in Table 1. Of the cases in which the exact localization was stated (36 nCP, 55 CPT), 52 samples derived from the lateral ventricle, 3 samples from the third ventricle and 36 samples from the fourth ventricle.

NCP samples were collected from 55 cases ranging in age from day 1 to 81 years (mean age 33.4 years). These samples were collected from routine autopsy brains (n=25) and from neurosurgical specimens (n=30). In the latter examples, surgery was performed for a variety of reasons (e.g. intracerebral hemorrhage, colloid cyst of the third ventricle) and the tissue sample contained choroid plexus tissue by chance. CPT tissue was analyzed from 88 tumor samples of 70 patients including 7 patients with tumor recurrences.

From each case, two representative samples (1.0 mm in diameter) from routinely paraffin-embedded specimens were selected to prepare tissue microarrays using a tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA). Additionally, in all cases of CPC (n=5), slides representing the whole area of the paraffin block were analyzed because of possible greater intratumoral heterogeneity. For the same reason, slides representing the whole area of the paraffin block were immunostained from some cases with nCP (n=16), CPP (n=5) and atypical CPP (n=5).

As Notch3 signaling has been reported to initiate CPT in embryonic mice (Dang et al., 2006) we additionally selectively investigated Notch3 expression (not Notch1, -2, -4 or Her2) in eleven fetal samples from choroid plexus achieved from routine autopsies (gestation age 9th to 39th gestational week).

Immunohistochemistry and evaluation

Slides were immunolabeled using antibodies against Notch1 (ab44986, monoclonal, Abcam, Cambridge, UK; 1: 100), Notch2 (polyclonal, LifeSpam BioSciences, Inc., Seattle, WA, USA; LS-B399, 1:25), Notch3 (1E4, monoclonal, 1:400, gift from A. Joutel, INSERM, Paris, France (Joutel et al., 2001)), Notch4 (polyclonal, LifeSpam BioSciences, Inc., Seattle, WA, USA; LS-B3498; 1:25) and Her2 (polyclonal, c-erbB-2, DakoCytomation, Glostrup, Denmark; A 0485; 1:500) using an automated immunohistochemistry slide staining system (BenchMark®, Ventana Medical Systems, Tucson, Az, USA). The automated standard protocol is

Table 1. Clinicopathological data on investigated cases.

nCP (postnatal)	WHO grade	n	sex (m/f)	age (mean; range)
		55	29 / 26	33.4 y. (day 1 – 81 y)
CPT	I-III	88	44 / 44	31.1 y. (0.1 – 74)
CPP	I	61	30 / 31	28.6 y. (0.2 – 72)
Atypical CPP	II	22	12 / 10	41.3 y. (0.2 – 74)
CPC	III	5	2 / 3	16.3 y. (0.1 – 37)

nCP, normal choroid plexus; CPT, choroid plexus tumor; CPP, choroid plexus papilloma; CPC, choroid plexus carcinoma.

based on an indirect biotin-avidin system that uses an universal biotinylated immunoglobulin secondary antibody and diaminobenzidine substrate. The sections were counterstained with haematoxylin. Negative controls consisted of sections incubated in the absence of the primary antibody.

As positive control we used human tissue samples from liver and hepatocellular carcinoma (Notch1-4), kidney (Notch1, -2 and -4), brain tissue (including choroid plexus) from two CADASIL patients (Notch3) and metastatic breast cancer (Her2).

Diagnosis of CPT was confirmed in all cases by positive immunostaining with antibodies Kir7.1 (a gift from S. Hirose, polyclonal rabbit, dilution 1:6000 (Nakamura et al., 1999)).

The numbers of Notch immunoreactive choroid plexus epithelial cells were evaluated using a 5-scale semiquantitative score: 0, <1%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, >75%. Thereby, membranous/cytoplasmic and nuclear Notch staining was separately evaluated. Her2 immunostaining was scored using the 4-scaled Dako scoring system: 0, 1+, 2+, 3+. This study was carried out according to the ethical guidelines of the University of Tuebingen, Germany (Project no. 311/2010B01).

Statistics

For analysis of the semiquantitative score values in the immunohistochemical studies, we used analysis of variance (ANOVA) with a subsequent student-t test for pairwise comparison of WHO tumor grades, sex-dependency, dependency on fixation times (nCP samples collected from routine biopsy vs. nCP samples collected from autopsy) and normal versus neoplastic tissue (each test separately for membranous/cytoplasmic and nuclear expression).

The sample size in all groups was sufficiently large for the central limit theorem to be applicable. This theorem asserts that the distribution of means is asymptotically normal, even if the individual varieties have a non-normal distribution (Everitt, 1996; Nakamura et al., 1999; Stevens, 1999). The mean scores are given together with the standard error (SE).

To test whether numbers of Notch or Her2 positive choroid plexus epithelial cells in nCP or in CPT depend on patient's age, an ordinal logistic regression was used. Testing for age-dependency of Notch expression was performed separately for membranous/cytoplasmic and nuclear staining for all 4 Notch receptors. Data on Notch3 expression in samples from fetal nCP was not considered for statistical analysis. Contingency table with subsequent Pearsons Chi-square test was used for correlation of Her2 expression in CPT and tumor recurrences (n=7).

For correlation of expression of the 4 Notch receptors and Her2 a multivariate analysis followed by pairwise comparisions was performed. Thereby, again membranous/cytoplasmic and nuclear Notch staining was separately compared to the generally membranous Her2 staining results. JMP IN 7.0 (SAS Institute, Cary, NC, USA) was used for statistical analysis (*P>0.05, **P>0.01, ***P>0.001).

Results

Expression of Notch1-4 and Her2 in control tissues

Tissue samples from liver (Notch1), kidney (Notch2 and Notch4), brain samples from two CADASIL patients (Notch3) and from metastatic breast cancer (Her2) served as positive controls (Fig. 1). As expected, Notch1, -2 and -4 showed a diffuse cytoplasmic/membranous staining in hepatocytes and epithelial cells from biliary epithelium (Notch1) and in epithelial cells from proximal tubule of kidney (Notch2, Notch4). Notch1 was additionally expressed in some nuclei from hepatocytes. Granular Notch3 expression was localized in the vessel walls and was notable not only in arterial vessels but also in several vessels with smaller diameter and thin vessel wall, according to veins and capillaries. Notch3 expression is mainly found in smooth muscle cells but also closely localized to endothelial cells and/or pericytes. A distinct membranous immunostaining for Her2 was observed in the majority of tumor cells of the breast cancer sample (Her2 Dako score 3+).

Notch expression in nCP and in CPT

Notch1, -2 and -4 were expressed in the vast majority of samples of postnatal nCP (100%, 72% and 100%, respectively) and CPT (97%, 99% and 100%, respectively). In contrast, no expression of Notch3 was observed in postnatal nCP and in CPT (0% each). All fetal tissue samples investigated lacked Notch3 expression in the CP, as well as in the adjacent brain tissue (gray and white matter) including ependymal cells (Notch1, -2 and -4 were not examined in fetal samples, compare materials and methods). In cases in which expression of Notch1-4 was investigated in both full slides and in TMAs, no difference in expression patterns were notable. Thus, there was no heterogeneity in Notch expression within tissue samples from a single patient notable, indicating that TMAs are suitable to examine Notch expression.

Notch expression at membranous/cytoplasmic localization

There was no significant difference in membranous/cytoplasmic expression of Notch1, -2 and -4 when comparing nCP samples collected from routine biopsy and nCP samples collected at autopsy, indicating that differences in fixation times did not affect Notch expression. Expression of Notch1 was significantly higher ($p<0.0001$) in nCP (mean score 3.900±0.194) compared to CPT (mean score 2.408±0.154). Similarly, Notch4 expression was significantly higher ($p=0.0046$)

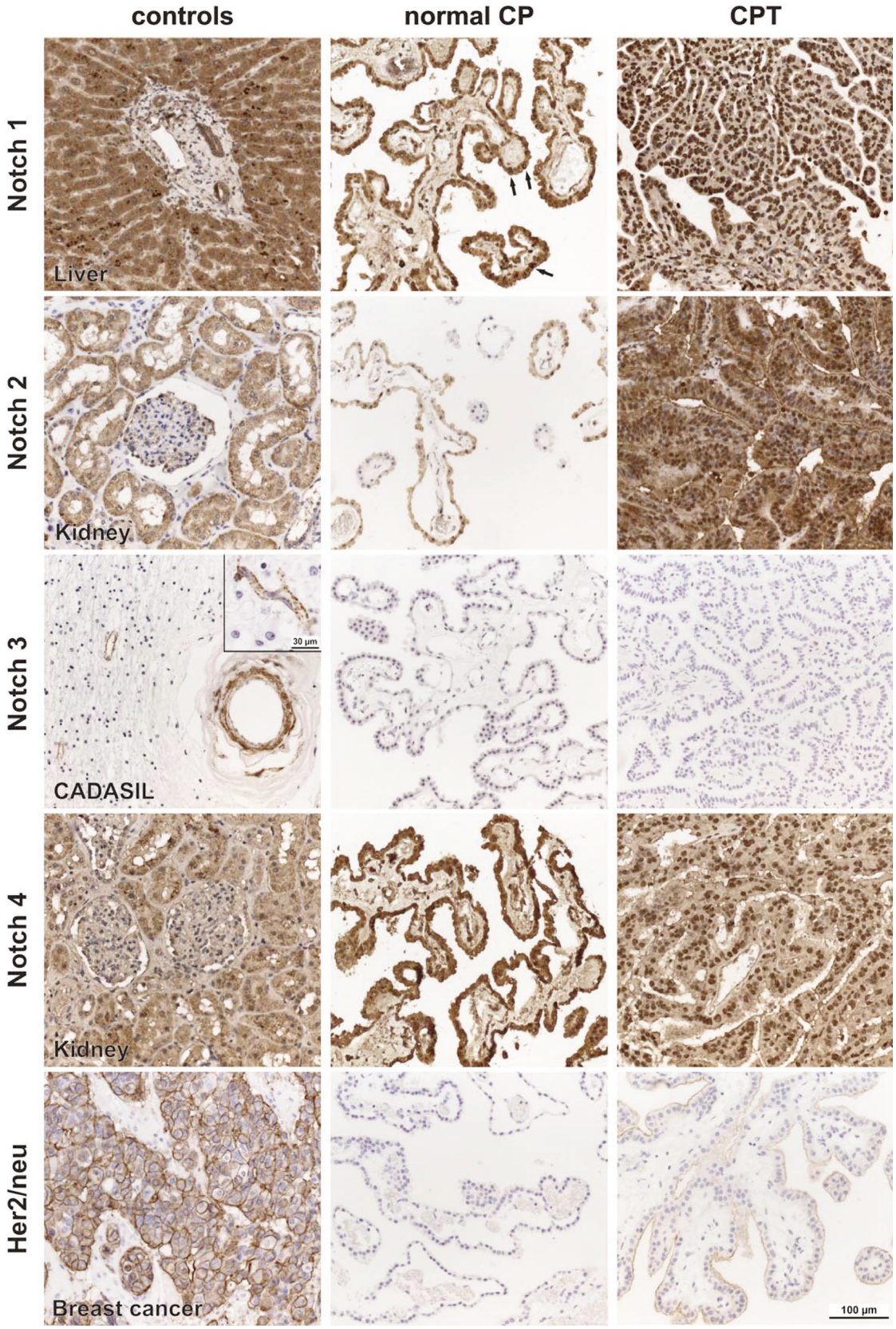

Fig. 1. Expression of Notch1-4 and Her2 in control tissues, normal CP and CPT. Representative immunostainings for Notch1-4 and Her2 in controls (left column), normal CP (middle column) and in CPT (right column) are shown. Note the predominant nuclear expression of Notch1, -2 and -4 in CPT in comparison to normal CP. x 200; inset x 1,000

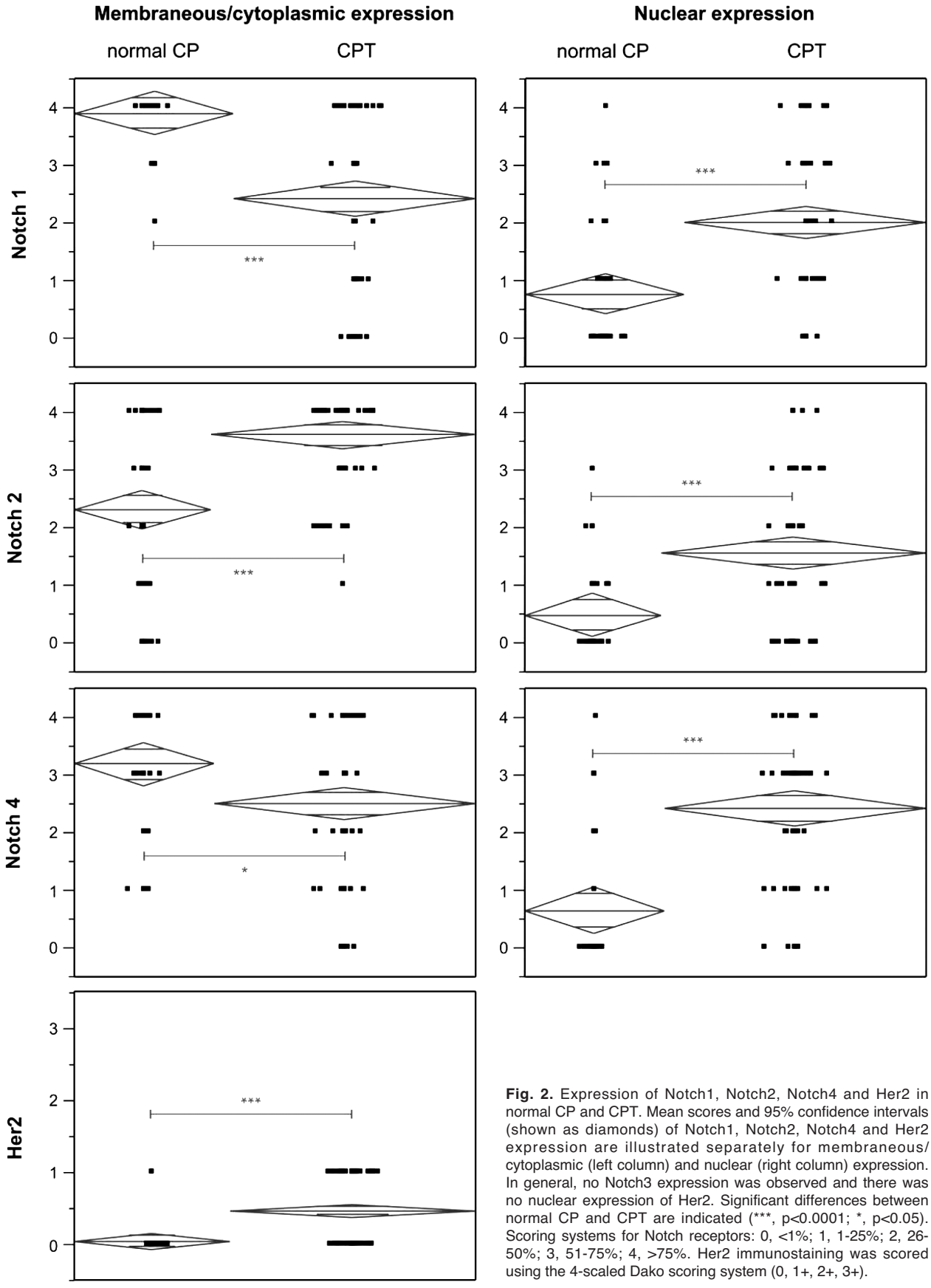

Fig. 2. Expression of Notch1, Notch2, Notch4 and Her2 in normal CP and CPT. Mean scores and 95% confidence intervals (shown as diamonds) of Notch1, Notch2, Notch4 and Her2 expression are illustrated separately for membraneous/cytoplasmic (left column) and nuclear (right column) expression. In general, no Notch3 expression was observed and there was no nuclear expression of Her2. Significant differences between normal CP and CPT are indicated (***, $p<0.0001$; *, $p<0.05$). Scoring systems for Notch receptors: 0, <1%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, >75%. Her2 immunostaining was scored using the 4-scaled Dako scoring system (0, 1+, 2+, 3+).

in nCP (mean score 3.186±0.194) compared to CPT (2.494±0.141). In contrast, Notch2 was significantly ($p<0.0001$) more expressed in CPT (3.596±0.121) compared to nCP (2.309±0.169). As stated above, no expression of Notch3 was detected in nCP or in CPT.

Notch expression at nuclear localization

There was no significant difference in nuclear expression of Notch 1, -2 and -4 when comparing nCP samples collected from routine biopsy and nCP samples collected at autopsy, indicating that differences in fixation times did not affect Notch expression. Expression of Notch1 (mean score 2.000±0.146), Notch2 (mean score 1.561±0.137) and Notch4 (mean score 2.420±0.151) was significantly higher in CPT compared to nCP ($p<0.0001$ each). No nuclear expression of Notch3 was detected either in nCP or in CPT.

Data on specificity, sensitivity, positive predictive value and negative predictive value when considering a cut-off value of 1% choroid plexus epithelial cells with nuclear Notch expression (score 0 versus score 1-4) in all cases of postnatal nCP and CPT, is listed in table 2.

Her2 expression in nCP and in CPT

Expression of Her2 was limited to cell membranes in 2/53 (4%) samples of nCP and 42/84 (50%) samples of CPT. No cytoplasmic or nuclear expression of Her2 was observed. According to the Dako scoring system, differences in Her2 expression were significant ($p<0.0001$) with higher levels in CPT compared to nCP. However, Her2 expression was generally low in nCP (mean Dako score 0.038±0.056) and in CPT (mean Dako score 0.464±0.045). Considering all cases of postnatal nCP and CPT, a cut-off value at Dako score 1+ (Dako score 0 versus score 1+, 2+ and 3+) distinguishes neoplastic and non-neoplastic CP with 96% specificity and 82% sensitivity and was associated with positive and negative predictive values of 97% and 79%, respectively. Furthermore, Her2 expression (Dako score) did not significantly differ between primary tumors and tumor recurrences (Pearsons Chi square: $p=0.6625$).

Correlation of Notch expression and Her2 expression with age, gender or grade of malignancy

Statistical evaluation of nCP samples showed no age-dependency and no sex-dependency either of Notch receptor expression or Her2 expression. Notch4 expression showed a tendency to increase with age but this effect did not reach a level of significance ($p=0,063$). Similarly, Notch4 expression in nCP was higher in males (mean score 3.4) compared to females (mean score 2.9) but again differences were not significant ($p=0.0968$).

For CPT, there was also no age-dependency and no sex-dependency either of Notch receptors expression or Her2 expression. Notch1 expression in CPT showed a tendency to increase with age but this effect did not reach a level of significance ($p=0,085$). Correlation analysis of receptor expression with WHO grade of tumors showed no significant correlation.

Discussion

Notch signaling in humans is associated with tumor formation in various types of tissue including carcinomas (Callahan and Egan, 2004; Gao et al., 2008; Bin et al., 2009; Zardawi et al., 2009; Srivastava et al., 2010; Serafin et al., 2011) and primary brain tumors (Puget et al., 2009; Xu et al., 2009; Milde et al., 2012).

In a rodent model Dang and coworkers reported that introduction of Notch3 into periventricular cells of embryonic mice causes the formation of CPT. In these tumors, Notch3 expression was noted mainly in a cytoplasmic localization but also in some nuclei of tumor cells (Dang et al., 2006). In contrast to the data in animal models, in our series on human samples (including samples from fetal CP) we could not detect any expression of Notch3 either in nCP or in CPTs. The lack of evidence for Notch3 signaling to play a role in the formation of the human CP during embryogenesis or in tumorigenesis of human CPT indicates that the functional role of Notch signaling might be different in humans and animals (Malinge and Crispino, 2010).

A shift from membranous/cytoplasmic to nuclear Notch expression during tumorigenesis has previously been reported in various types of human tissues for all 4 Notch receptors (Cobellis et al., 2008; Yoon et al., 2011). Likewise, we observed a significant increase in nuclear expression of Notch1, Notch2 and Notch4 in CPT compared to nCP, indicating an enhanced role of nuclear Notch signalling (canonical pathway) in tumorigenesis of human CPT. A nuclear expression of Notch1, -2 or -4 in more than 25% of CP epithelial cells (score 2-4) indicates neoplastic transformation. Interestingly, such a shift to a predominant nuclear expression has also been reported for Notch3 in extrahepatic cholangiocarcinomas and gallbladder carcinomas (Yoon et al., 2011). However, Notch3 was not expressed in any samples of nCP or CPT, either in the nucleus or in a membranous/cytoplasmic localization.

Expression of Her2 according to the Dako scoring

Table 2. Statistical values for nuclear Notch expression

	Notch1	Notch2	Notch3	Notch4
Specificity	62%	69%	-	74%
Sensitivity	82%	68%	-	84%
Positive predictive value	77%	82%	-	86%
Negative predictive value	69%	52%	-	71%

Statistical values when comparing nuclear Notch expression in nCP and CPT considering a cut-off value of 1% CP epithelial cells with nuclear Notch expression.

system (HercepTestTM) is generally low in nCP and in CPT. Although the mean Dako score for Her2 expression is significantly higher in CPT, the maximum Dako score identified in nCP and in CPT is only 1+ each. According to the interpretation criteria for HercepTestTM in breast cancer all investigated samples of nCP and CPT have to be interpreted as "negative" (score 0 or 1+). Therefore, no Her2 gene amplification is to be expected in CPT and there is no evidence for Her2 expression to have a prognostic or therapeutic relevance in CPT. In our series of CPT, including 7 tumor recurrences, there was no correlation between Her2 expression and tumor recurrence. As the mean Dako score is significantly higher in CPT, a membranous Her2 expression (among other markers such as EAAT1) may be helpful in distinguishing neoplastic CP from nCP (Beschorner et al., 2009).

In therapeutic oncology, besides drugs targeting Her2 such as trastuzumab (Herceptin®), therapeutic antibodies targeting Notch receptors directly (Wu et al., 2010; Sharma et al., 2012) and other drugs affecting the Notch signaling pathway such as gamma secretase inhibitors (Kopan and Ilagan, 2009; Fouladi et al., 2011) have promising potential. These studies also emphasize the therapeutic promise in targeting individual Notch receptors or Notch receptor signaling pathways independently. For example, the expression pattern of Notch4 appears to be considerably more restricted than that of Notch1, suggesting that agents targeting Notch4 may have less systemic toxicity than agents targeting Notch1 (Rizzo et al., 2008).

In this study we were able to demonstrate that Notch1 and Notch4 are expressed in nCP as well as in CPT. Although the subcellular localization of Notch1 and Notch4 expression significantly differs between normal (predominant membranous/cytoplasmic expression) and neoplastic CP epithelium (predominant nuclear expression) our overall data indicates that Notch1 and Notch4 are not optimal therapeutic targets in CPT. In contrast, membranous/cytoplasmic and nuclear expression of Notch2 is significantly higher in CPT compared to nCP. Therefore, therapeutic strategies targeting Notch2 signaling in CPT seem to be more promising than approaches on Notch1 or Notch4 signaling. In contrast to the experimental study by Dang and coworkers (Dang et al., 2006) our data show no evidence for Notch3 signaling to play a role in CPT formation in humans or to be a potential therapeutic target in human CPT.

It is now well established that Notch receptor activation is mediated by a sequence of proteolytic events, and turning Notch signaling "off" pharmacologically via γ-secretase inhibition has become a common experimental tool. More recent knowledge of Notch pathway core components and further understanding of the sequences of proteolytic events during activation now make it possible to transiently turn endogenous Notch signaling "on" whenever needed for therapeutic or tissue-engineering purposes (Kopan and Ilagan, 2009).

Recently, in a pediatric brain tumor consortium study MK-0752, a gamma secretase inhibitor, was reported in a phase I trial to be well-tolerated in children with recurrent CNS malignancies, including CPC (Fouladi et al., 2011). RO4929097, another gamma secretase inhibitor, was well-tolerated in a phase I study on metastatic or locally advanced solid tumors (Tolcher et al., 2012). However, RO4929097 showed only minimal single agent activity at the study dose and schedule in a phase II trial on metastatic colorectal cancer (Strosberg et al., 2012).

It was long believed that pathological changes in CADASIL, especially Notch3 deposition, only affect arteries, which is already indicated by the name of the disease (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy). However, previous studies reported granular osmophilic material (GOM) and/or Notch3 expression also in veins (Rafalowska et al., 2004; Saiki et al., 2006) and capillaries (Rafalowska et al., 2003; Joutel et al., 2010; Lewandowska et al., 2010) of CADASIL patients. In accordance with these reports, we observed distinct Notch3 immunoreactivity in brain tissue from CADASIL cases, not only in arteries, but also in veins and capillaries.

In summary, our data indicates that Notch1, 2 and 4 but not Notch3, are expressed in CPT and a shift from cytoplasmic/membranous expression to the nuclei indicates neoplastic transformation of the choroid plexus epithelium. For future anti-Notch therapy as a possible treatment strategy in CPT it should be considered, that strategies targeting Notch2 with maximum differences between normal and neoplastic tissue might be more effective than approaches targeting Notch1 and Notch4. Finally, there seems to be no direct role for Notch3 signaling in CP development or CPT formation in humans.

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