COX/mPGES-1/PGE2 pathway depicts an inflammatory-dependent high-risk neuroblastoma subset

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The majority of solid tumors are presented with an inflammatory microenvironment. Proinflammatory lipid mediators including prostaglandin E2 (PGE2) contribute to the establishment of inflammation and have been linked to tumor growth and aggressiveness. Here we show that high-risk neuroblastoma with deletion of chromosome 11q represents an inflammatory subset of neuroblastomas. Analysis of enzymes involved in the production of proinflammatory lipid mediators showed that 11q-deleted neuroblastoma tumors express high levels of microsomal prostaglandin E synthase-1 (mPGES-1) and elevated levels of PGE2. High mPGES-1 expression also corresponded to poor survival of neuroblastoma patients. Investigation of the tumor microenvironment showed high infiltration of tumor-promoting macrophages with high expression of the M2-polarization markers CD163 and CD206. mPGES-1-expressing cells in tumors from different subtypes of neuroblastoma showed differential expression of one or several cancer-associated fibroblast markers such as vimentin, fibroblast activation protein α, α smooth muscle actin, and PDGFR receptor β. Importantly, inhibition of PGE2 production with diclofenac, a nonselective COX inhibitor, resulted in reduced tumor growth in an in vivo model of 11q-deleted neuroblastoma. Collectively, these results suggest that PGE2 is involved in the tumor microenvironment of specific neuroblastoma subgroups and indicate that therapeutic strategies using existing anti-inflammatory drugs in combination with current treatment should be considered for certain neuroblastomas.

mPGES-1 | PGE2 | neuroblastoma | tumor microenvironment | cancer-associated fibroblasts

Neuroblastoma is the most common and deadliest tumor of childhood. Although the survival of children with neuroblastoma has improved during the last decade, patients with high-risk disease still have a poor prognosis, despite advanced and intensive treatments, with overall survival rates less than 40% (1). The International Neuroblastoma Risk Group (INRG) classification system defines neuroblastoma risk groups as low, intermediate, and high, based on age at diagnosis, histology, and genetic aberrations (2). Among high-risk neuroblastomas, amplification of the neuroblastoma MYC (MYCN) oncogene is the most frequent genetic aberration, seen in 30–40% of the patients. Another common genetic change in the high-risk group is deletion of the long arm of chromosome 11 (11q-deletion). Deletion of 11q occurs in tumors with multiple genetic aberrations and chromosome instability but commonly without MYCN-amplification and therefore is a useful prognostic marker in adverse-stage tumors lacking MYCN-amplification (3). These patients are often older at disease onset and have slow disease progression but often develop therapy resistance and have poor clinical outcome. Among low- and intermediate-risk neuroblastomas are the so-called “special neuroblastomas” (4S) that show a metastatic phenotype but are associated with spontaneous regression and a survival rate of 90% (1, 4, 5).

Prostaglandins are bioactive lipids involved in many biological processes both in physiological processes e.g., blood pressure, smooth muscles contraction, and protection of the intestinal mucosa, and in pathological conditions such as autoimmune diseases and cancer (6). Prostaglandins are formed by the conversion of arachidonic acid to prostaglandin H2 (PGH2) by the cyclooxygenases COX-1 and COX-2, followed by further processing by terminal enzymes, the prostaglandin synthases. Prostaglandin E2 (PGE2) is a proinflammatory and immunomodulatory lipid mediator formed from PGH2 by microsomal prostaglandin E synthase 1 (mPGES-1). Elevated levels of mPGES-1 and its enzymatic product PGE2 have been found in several different cancers, including colon cancer (7–9), nonsmall cell lung cancer (10), and prostate cancer (11, 12). PGE2 signaling contributes to increased proliferation (13, 14) and invasiveness (15) of cancer cells, stimulates tumor angiogenesis (16, 17), inhibit apoptosis (18), induces chemoresistance (19), and mediates suppression of anti-tumor immunity (20, 21). Because tumor-promoting inflammation has been included as one of the hallmarks of cancer (22), chronic inflammation and its impact on tumorigenesis in adult tumors have been immensely investigated.

Less is known about the inflammatory component in childhood tumors; however, in a recent study, Ashgarzadeh et al. (23) showed that infiltration of immunosuppressive M2-polarized macrophages in metastatic MYCN-nonamplified neuroblastoma tumors contributed to the metastatic phenotype and worsened

Significance

Cancer-related inflammation promotes progression and therapy resistance in tumors of adulthood. Knowledge concerning the significance of inflammation in childhood malignancies has been limited. Neuroblastoma is an embryonal tumor of early childhood with poor prognosis despite intensified therapy, and biological understanding is necessary to develop novel therapies. We found high-risk neuroblastoma, in particular the therapy-resistant subset with chromosome 11q-deletion, to be inflammatory driven and characterized by high expression of the COX/microsomal prostaglandin E synthase-1 (mPGES-1)/prostaglandin E2 (PGE2) pathway that correlates with metastatic stage and poor clinical outcome. We further detected infiltrating cancer-associated fibroblasts expressing mPGES-1, the essential enzyme for synthesis of PGE2, promoting tumor growth, angiogenesis, and metastatic spread. Treatment targeting this inflammatory pathway provides a therapeutic option for neuroblastoma and other cancers.


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the outcome of these patients. Cancer-associated fibroblasts (CAFs) also have been found to contribute to tumor development and metastasis (24), and in a study by Zeine et al. (25) a high number of CAFs was found to correlate with more aggressive Schwannian stroma-poor neuroblastoma tumors.

We previously reported the effect of anti-inflammatory drugs on tumor growth in preclinical in vivo models of neuroblastoma (26–28). We also have found high expression of the PGE2 receptor EP2 in neuroblastoma tumor tissue as well as effects of PGE2 on neuroblastoma cell growth in vitro (29). These results suggest that inflammatory processes are important for neuroblastoma growth. Therefore we investigated the importance of proinflammatory prostaglandins and their enzymes in neuroblastoma subgroups.

Results

mPGES-1 mRNA Expression in Primary Neuroblastoma. To study the expression levels of mPGES-1, COX-1, and COX-2 in primary neuroblastoma, quantitative real-time PCR analysis of tumor tissue was performed. When the relative expression was analyzed, significantly higher expression of mPGES-1 mRNA was seen in the 11q-deleted tumors than in low-risk tumors alone (P = 0.04), and there was a strong tendency toward a difference in mPGES-1 mRNA expression between 11q-deleted tumors vs. MYCN-amplified and low-risk tumors considered together (P = 0.06, Mann–Whitney; P = 0.04, t test). The expression of COX-1 was significantly different in 11q-deleted tumors and low-risk tumors (P = 0.03) and in 11q-deleted tumors compared with MYCN-amplified and low-risk tumors considered together (P = 0.02). No significant differences were found for COX-2 (Fig. L4).

Using publicly available expression cohorts, we analyzed mPGES-1 gene expression levels and its correlation to overall survival in patients with International Neuroblastoma Staging System (INSS) stage 4 tumors. Patients with tumors expressing high levels of mPGES-1 had an overall survival of only 10%, compared with 62% in patients with tumors expressing low levels of mPGES-1 (P = 9.6e-04) (Fig. 1B).

Prostaglandin Profiling of Neuroblastoma Tumors. Prostaglandin levels were measured by LC-MS/MS analysis of liquid–liquid extracted tissue homogenates from 29 primary neuroblastoma samples (four 11q-deleted, eight MYCN-amplified, and 17 low-risk tumors; Table S1). There were significantly higher levels of PGE2 in the 11q-deleted tumors than in the MYCN-amplified tumors (P = 0.02) or in the low-risk tumors (P = 3.0e-04) (Fig. 1C). No significant differences were found in the levels of other prostanoids analyzed (Fig. S1 and Table S2).

Immunohistochemical Analysis of Prostaglandin Synthases. The expression of enzymes involved in synthesis of prostaglandins in neuroblastoma was analyzed using immunohistochemistry (IHC). COX-1, COX-2, mPGES-1, lipocalin-type prostaglandin D synthase (L-PGDS), hematopoietic-type prostaglandin D synthase (H-PGDS), and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) were analyzed in tumors from 11 neuroblastoma patients (four 11q-deleted, three MYCN-amplified, and four low-risk tumors; Fig. 2 and Fig. S2). Expression of mPGES-1 was found in all tumors tested, with significantly higher levels in the 11q-deleted tumors than in the low-risk tumors alone (P = 0.02) or in the MYCN-amplified and low-risk tumors considered together (P = 0.004). mPGES-1 expression was confirmed with IHC in additional 11q-deleted tumors (Fig. S3) and with Western blot. Western blot analysis clearly showed higher levels of mPGES-1 in the 11q-deleted tumors than in the MYCN-amplified and low-risk tumors. COX-1 was detected in one of the 11q-deleted tumors and in tumors from patients with low-risk and 11q-deleted tumors. Of all tumors analyzed, COX-2 was detected in only one of the MYCN-amplified tumors (Fig. S3). Consistent with Western blot analysis, IHC analysis showed that there was more expression of COX-1 than COX-2 in the neuroblastoma tumors, and the difference between COX-1 and COX-2 expression was more evident in the 11q-deleted tumors, but no significant difference between the tumor subgroups was found for either COX-1 or COX-2 (Fig. 2B). Both prostaglandin D2 (PGD2) synthases were present in significantly higher levels in low-risk tumors than in 11q-deleted tumors (L-PGDS, P = 6.0e-04; H-PGDS, P = 0.03). Elevated levels of H-PGDS also were detected in MYCN-amplified tumors as compared with 11q-deleted tumors (P = 0.02) (Fig. S2). The 11q-deleted tumors expressed significantly lower levels of 15-PGDH, an enzyme responsible for PGE2 and PGD2 degradation, than did either low-risk tumors alone (P = 0.005) or MYCN-amplified and low-risk tumors taken together (P = 0.008).

Because of the relatively high levels of COX-1 compared with COX-2 in the neuroblastoma tumors, double staining of mPGES-1 and COX-1 was performed. In one of the three tumors tested, there was considerable overlap between COX-1 and mPGES-1 was observed. In two of the three tumors only single cells expressed both enzymes, but cells expressing the respective enzymes were in proximity to each other (Fig. 2C).

IHC Analysis of the Tumor Microenvironment in Relation to mPGES-1 Expression. IHC analysis of mPGES-1 in neuroblastoma samples showed positive staining of mPGES-1 in nontumorigenic cells, suggesting that stromal or tumor-infiltrating cells express mPGES-1 (Fig. 2A). We therefore performed IHC analysis using an mPGES-1 antibody in combination with antibodies detecting different immune cells, stromal and endothelial cells, and fibroblasts known to be part of the tumor microenvironment (Table 1). The double staining was performed on a tumor from one patient in each of the subgroups; we designated these tumors “NB1” (untreated, MYCN-amplified, INSS stage 2, unfavorable prognosis), “NB4” (untreated, low-risk, INSS 4S, favorable prognosis), and tumor “NB43” (pretreated, 11q-deleted, INSS stage 4, unfavorable prognosis).

Disialoganglioside GD2 is expressed almost exclusively on tumors of neuroectodermal origin and is used as therapeutic target in neuroblastoma (30). Double staining of GD2 with mPGES-1 showed a considerably different staining pattern. No coexpression of GD2 and mPGES-1 was found in any of the three tumors analyzed (NB1, NB4, or NB43). The expression was mutually exclusive, with a clear compartmentalization of cells expressing the respective proteins (Fig. 3A).

Fig. 1. mPGES-1 expression and PGE2 levels are associated with 11q-deleted neuroblastoma. (A) Expression of mPGES-1, COX-1, and COX-2 mRNA in neuroblastoma tissue samples was measured by the TaqMan Gene Expression Assay. Expression of mPGES-1 and COX-1 was significantly higher in 11q-deleted tumors than in low-risk tumors (P = 0.04 and P = 0.05, respectively). No difference in COX-2 levels was found. (B) Analysis of an expression cohort shows high expression of mPGES-1 associated with worse overall survival in metastatic neuroblastoma (INSS stage 4). (C) PGE2 levels in neuroblastoma tissue samples as measured by LC-MS/MS were significantly higher in 11q-deleted tumors than in MYCN-amplified (NMA) (P = 0.02) and low-risk tumors (P = 3.0e-04). Bars in A and C represent median values.
Because macrophages are a sign of inflammation, are a typical source of mPGES-1 expression (31), and are present in tumor microenvironments as tumor-associated macrophages, double staining of mPGES-1 in combination with a pan-macrophage marker (CD68) and a marker for M2-polarized macrophages (CD163) was used. All analyzed tumors showed widespread expression of CD68 throughout the sections, thus showing the presence of macrophages (Fig. 3B). Interestingly, no colocalization of mPGES-1 and CD68 was seen. CD163 was also expressed abundantly throughout the tumor sections, but no colocalization between mPGES-1 and CD163 was found in any of the neuroblastoma samples analyzed (Fig. S4). To consider the extent of macrophage infiltration and macrophage polarization, tumors from three patients in each subgroup were stained with CD163, CD206 (M2-polarization), and MHC class II (MHCII; higher expression in M1). There were significantly more M2-polarized macrophages in 11q-deleted and MYCN-amplified tumors than in low-risk tumors (see Fig. S5), suggesting a more immunosuppressive microenvironment in high-risk neuroblastoma.

Apart from macrophages, other myeloid cells, including myeloid-derived suppressor cells (MDSCs), are known to infiltrate tumor tissue. To cover all myeloid cells, double staining of mPGES-1 with CD11b was performed, but no colocalization was seen (Fig. S4).

To determine if dendritic cells contribute to mPGES-1 expression in neuroblastoma, double staining of mPGES-1 and CD11c was performed, but no colocalization was found. However, in the NB4 and NB43 tumors the CD11c+ cells were found only in areas with mPGES-1+ cells (Fig. S4). In the MYCN-amplified tumor, NB1, there was an overall pattern of weak CD11c staining, and no positive CD11c staining was detected in the proximity of mPGES-1+ cells.

Next we studied lymphocyte infiltration in the proximity of mPGES-1+ cells (Fig. S4). CD3+ T cells were present in all tumors to a varying extent. The NB1 and NB4 tumors had no T cells in mPGES-1+ areas. Tumor NB1 showed few CD3+ cells in mPGES-1+ areas, whereas CD3+ T cells were abundant in presence in the mPGES-1+ expressing areas of the NB4 tumor. These T cells also were CD4+4. CD3+ T cells were abundant throughout the NB4 tumor and were slightly enriched in mPGES-1+ areas. Interestingly, B cells were abundant only in the NB4 tumor and only in proximity to mPGES-1+ expressing cells (Fig. S4).

Subsequently we studied the contribution of mPGES-1 expression from endothelial cells. Double staining of mPGES-1 and the endothelial cell marker CD31 showed colocalization with mPGES-1+expressing cells in some areas of the NB4 tumor, but no colocalization was detected in the NB1 or NB43 tumors (Fig. S4).

To determine if the mPGES-1+ expressing cells could be of mesenchymal origin, we performed double staining of mPGES-1 and vimentin (Fig. 3C). A majority of the mPGES-1+ cells also showed positive staining for vimentin in the NB4 tumor. In the NB1 tumor the majority of cells were positive for vimentin, including cells expressing mPGES-1 (Fig. 3C). The NB43 tumor showed weak vimentin staining, and only a few of these cells coexpressed mPGES-1.

Because the majority of mPGES-1+ cells in the NB1 and NB4 tumors also expressed vimentin, we performed IHC analysis with markers for CAFs, i.e., fibroblast activation protein α (FAP), PDGF receptor β (PDGFRβ), fibroblast-specific protein 1 (FSP-1), a smooth muscle actin (αSMA), and PDGF receptor α (PDGFRα) (Fig. 4). In the NB1 tumor there was no positive staining of cells for mPGES-1 and CAF markers. Cells expressing mPGES-1 exhibited strong positive staining for FAP and αSMA and weak positive staining for PDGFRβ, whereas none of the cells were positive for PDGFRα or FSP-1 (Fig. 4). In the NB4 tumor there was colocalization of mPGES-1 with PDGFRα, PDGFRβ, and FSP-1 but no colocalization with FAP and αSMA (Fig. 4). Only weak positive staining for CAF markers was detected in the NB43 tumor, and only FSP-1 showed colocalization with mPGES-1.

**COX Inhibition of an in Vivo Xenograft Mouse Model of 11q-Deleted Neuroblastoma.** The effect of COX inhibition on neuroblastoma tumors in vivo was assessed in an 11q-deleted neuroblastoma cell line (32) (SK-N-AS) xenograft mouse model treated with diclofenac (Fig. 5). Tumor volume was measured and compared with untreated animals. A significant reduction in tumor growth was found for diclofenac-treated animals compared with control animals on day 8 ($P = 0.01$) and day 9 ($P = 0.008$) (Fig. S4). Endogenous levels of PGE2 were measured in the tumors using LC-MS/MS of solid-phase extracted tumor tissue. There was a significant decrease of PGE2 in tumors from diclofenac-treated animals compared with controls ($P = 0.04$) (Fig. S5B).
Table 1. Localization of different components of the tumor microenvironment in relation to mPGES-1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Marker</th>
<th>NB1, NMA</th>
<th>11q-deleted, MYCN-amplified</th>
<th>Low-risk, MYCN-amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroblastoma</td>
<td>GD2</td>
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<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myeloid cells</td>
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<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>CD163</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<td></td>
<td>CD11b</td>
<td>No</td>
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<tr>
<td></td>
<td>CD11c</td>
<td>No</td>
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<tr>
<td>T cell</td>
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<tr>
<td>B cell</td>
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<td>No</td>
</tr>
<tr>
<td>Epithelial cells</td>
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<td>No</td>
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</tr>
<tr>
<td>Mesenchymal</td>
<td>Vimentin</td>
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<td>No</td>
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</tr>
<tr>
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<td>uSMA</td>
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<td></td>
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<td>No</td>
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<td></td>
<td>FSP-1</td>
<td>No</td>
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</table>

Colocalization was scored as negative (no) or positive (yes) in tumors from three patients, one from each subgroup [11q-deleted tumor NB43, MYCN-amplified tumor NB1], and low-risk (tumor NB4).

Discussion

The involvement of chronic inflammation in the initiation and progression of adult tumors of epithelial origin has been well established (6). Much less is known about the contribution of inflammation and proinflammatory mediators in solid childhood cancers, and we therefore investigated the involvement of prostaglandins in different subsets of neuroblastoma.

We found that mPGES-1 is expressed in human primary neuroblastoma samples. Expression of mPGES-1 was present in all tumors tested and was significantly higher in the 11q-deleted subset of high-risk tumors than in low-risk tumors on both the mRNA (Fig. L4) and the protein level (Fig. 2). Our lipid analysis showed significantly higher levels of PGE2, the enzymatic product of mPGES-1, in the 11q-deleted subset than in either MYCN-amplified or low-risk tumors (Fig. 1C), confirming the observations in the IHC analysis. In addition, significantly lower levels of 15-PGDH, the enzyme responsible for metabolizing PGE2, were detected in the 11q-deleted tumors, possibly contributing to the high PGE2 levels found in these tumors (Fig. S2). All patients with high-risk neuroblastomas are subjected to the same induction treatment before surgical excision of the tumor (Table S3) (33), and all patients had at least 2 wk off chemotherapy before surgery. Any effect of the chemotherapeutic drugs on prostaglandin levels would have affected the patients in both of the high-risk subgroups (i.e., patients with 11q-deleted tumors and those with MYCN-amplified tumors) similarly.

The mPGES-1 staining pattern in the IHC analysis suggested that cells located in the tumor microenvironment, and not tumor cells, are responsible for the production of PGE2. To confirm this suggestion, double staining with mPGES-1 and GD2, a neuroblastoma tumor cell marker, was performed. In all three tumors analyzed a clear discrepancy in staining pattern was seen. The areas with mPGES-1 expression and GD2-expressing cells were mutually exclusive, indicating that PGE2 is produced by cells in proximity to tumor cells but not by the tumor cells themselves. To our surprise, low levels of COX-2 were detected in the majority of neuroblastoma samples analyzed (Fig. 2), but the pattern of COX-1 staining was similar to that of mPGES-1. Double staining with mPGES-1 and COX-1 showed coexpression of both enzymes in some cells. We also detected cells with only COX-1 or mPGES-1 expression, perhaps because the enzyme was present at levels below the limit of detection for IHC analysis. However, COX-1 also has been shown to exert paracrine distribution of PGH2 to mPGES-1 (34). PGH2, which is needed for conversion to PGE2, also could be supplied from circulating cells expressing COX-1 or COX-2.

As discussed by Pistoia et al. (35), there is an immunosuppressed tumor microenvironment in neuroblastoma that potentially enables tumor cells to evade host immune responses. This immunosuppressive state has been shown in human tumor samples by the infiltration of M2-polarized macrophages (23) and in neuroblastoma mouse models by the infiltration of MDSCs (26, 36). Our data show predominance toward M2-polarization of the tumor-infiltrating macrophages (i.e., tumor-associated macrophages) in both 11q-deleted tumors and MYCN-amplified tumors, with elevated levels of CD206 and CD163, respectively (Fig. S3), supporting the findings by Aghasharzead et al. (23). Macrophages did not stain positive for mPGES-1 in the tumor samples, double staining with the dendritic cell marker CD11c was performed. No colocalization was found; however, CD11c-expressing cells were in cell-to-cell contact with the mPGES-1–expressing cells in the NB4 tumor and to some extent in the NB43 tumor (Fig. S4). This finding led us to explore further different populations of immune cells present in the tumor samples in relation to mPGES-1+ cells. Strikingly, in the 4S tumor NB4 there were CD3+ CD4+ T cells and CD20+ B cells only in clusters together with mPGES-1+ cells. Outside these immunological hotspots, no B cells or T cells were present. In the NB43 tumor, CD3+ CD4+ T cells were widespread in the tumor sections, although they were slightly more concentrated in the mPGES-1+ expressing cell clusters. Nonetheless, essentially no CD20+ B cells could be found in either the NB1 or the NB43 tumor. Spontaneous regression is an intriguing feature of 4S tumors, and the finding that only the 4S neuroblastoma sample had B cells is interesting and should be investigated further.

Endothelial cells have been shown to express mPGES-1 and to secrete PGE2 (31). Co-localization between mPGES-1 and CD31 was detected in the 4S neuroblastoma sample NB4 (Fig. S4). In the NB4 tumor and in the MYCN-amplified NB1 tumor, we also identified colocalization of mPGES-1 with vimentin. Vimentin is an intermediate filament found in nerve cell progenitors and also in cells of mesenchymal origin and is used as a
marker of epithelial-to-mesenchymal transition (EMT), a process promoting tumor metastasis (37). In a recent publication, epithelial tumor cells induced with EGF led to overexpression of mPGES-1 concomitant with increased vimentin expression and down-regulation of E-cadherin, initiating the phenotypic change of EMT (38). Both the NB1 and the NB4 tumors had relatively high vimentin expression, whereas only sporadic vimentin expression was found in the 11q-deleted tumor NB43, possibly because of chemotherapeutic treatment of this tumor before resection (Tables S1 and S3). Because vimentin also is expressed in fibroblasts, we looked at CAFs. In a recent study Alcolea et al. (39) showed that head and neck squamous cell carcinoma cells stimulate both dermal and tumor-derived fibroblasts to express mPGES-1, and they also observed PGE2 release in tumor-adjacent mucosa (39). Erez et al. (40) showed that CAFs educated by tumor cells mediated tumor-enhancing inflammation in an NF-κB-dependent manner. In line with these results, we found that mPGES-1+ cells in all three tumors analyzed (NB1, NB4, and NB43) expressed one or more markers for CAFs. The MYCN-amplified tumor NB1 coexpressed mPGES-1 and CAF markers such as vimentin, FAP, αSMA, and PDGFRβ but not FSP-1 and PDGFRα (41). Although mPGES-1+ cells in NB4 expressed vimentin, PDGFRα, PDGFRβ, and FSP-1, no colocalization with FAP or αSMA was detected. In the 11q-deleted tumor NB43, FSP-1 was the only CAF marker present in mPGES-1+ cells, and the expression of CAF markers was generally low (Fig. 4). Even though referred to as one population of cells, CAFs are heterogeneous in their function and origin as well as in their expression of markers (41, 42), possibly reflecting the differential expression of CAF markers seen in the neuroblastoma tumors. Recently, CAFs also have been found to contribute to an immunosuppressive tumor microenvironment (43).

Next we wanted to investigate the effect of PGE2 inhibition on tumor growth in an in vivo model of 11q-deleted neuroblastoma. Treatment with diclofenac, a nonselective COX inhibitor, resulted in reduced tumor growth. A reduction in PGE2 in the tumors was confirmed also. Based on our data, we propose that mPGES-1 is expressed in subpopulations of CAFs; therefore only a fraction of cells in the whole tumor produce PGE2, explaining the relatively low levels of PGE2 found. Still, the decrease in PGE2 production resulting from diclofenac treatment is enough to have a significant effect on tumor progression in the in vivo 11q-deleted model, suggesting that targeting PGE2 could be of benefit for children with aggressive, chemoresistant 11q-deleted neuroblastomas, as was indicated recently for bladder cancer (19).

In conclusion, we have identified an activated COX/mPGES-1/PGE2 pathway in 11q-deleted neuroblastoma with high expression of mPGES-1, low expression of 15-PGDH, and elevated levels of PGE2 as compared with MYCN-amplified and low-risk tumors. Analysis of expression cohorts revealed a worse outcome for high-risk patients (INSS stage 4) with high mPGES-1 expression. In addition, COX inhibition in an in vivo model of 11q-deleted neuroblastoma resulted in reduced tumor growth. These findings collectively suggest that PGE2 signaling in 11q-deleted high-risk tumors could be targeted using existing nonsteroidal anti-inflammatory drugs or by developing new compounds directly targeting mPGES-1. In addition there are convincing observations that mPGES-1/PGE2 may play a role in the complex network of the tumor microenvironment in general and specifically in CAFs (39, 44), further strengthening the suggestion that mPGES-1 should be targeted in neuroblastoma and in other tumors with activated COX/mPGES-1/PGE2 signaling.

Materials and Methods

The animal experiment was approved by the Stockholm North Animal Research Committee (approval N231/14) in accordance with the Animal Protection Law (SJVFS 2012:26), the Animal Protection Regulation (SFS 1988:539), and the Regulation for the Swedish National Board for Laboratory Animals (SFS 1988: 541).

Detailed materials and methods are provided in SI Materials and Methods.

Patient Material. Neuroblastoma tumor tissue was obtained from the Karolinska University Hospital. Ethical approval was obtained by the Stockholm Regional Ethical Review Board and the Karolinska University Hospital Research Ethics Committee (approval nos. 2009/1369-31/1 and 03/736). Tumor and patient characteristics are summarized in Table S1. Thirty-two neuroblastoma samples representing all clinical subsets were used.

Prostaglandin Profiling. Approximately 40 mg of human primary tumor tissue from 29 individuals was used in the analysis. Liquid–liquid extraction and analysis by LC-MS/MS was performed essentially as described previously (26).

![Fig. 4. Cellular localization of mPGES-1 expression in neuroblastoma tumors.](image)

Three human neuroblastoma tumors, NB1 (MYCN-amplified), NB43 (11q-deleted), and NB4 (low-risk 4S), were stained for mPGES-1 (green) and FAP (A), PDGFRα (B), FSP-1 (C), αSMA (D), or PDGFRβ (E), markers for cancer-associated fibroblast (CAFs), in red. All sections were counterstained with the nuclear dye Hoechst (blue). Colocalization was seen for FAP, αSMA, and PDGFRα in the NB1 tumor. Colocalization of PDGFRα, PDGFRβ, and FSP-1 also was seen in the NB4 tumor. In the NB43 tumor mPGES-1 colocalized with FSP-1. Taken together, these results suggest that CAFs are a source for mPGES-1 expression and PGE2 production.

![Fig. 5. COX inhibition reduces PGE2 levels and reduces tumor growth in vivo.](image)

Nude mice were inoculated with SK-N-AS neuroblastoma cells harboring an 11q-deletion and were treated with diclofenac (250 mg/L drinking water) for nine consecutive days. (A) Tumor volumes were measured each day, and a significant difference between diclofenac-treated and control mice was seen on day 8 (P = 0.01) and day 9 (P = 0.008). (B) PGE2 was measured in the tumors using solid-phase extraction and LC-MS/MS analysis. There was a significant decrease in PGE2 in tumors from diclofenac-treated mice compared with control mice (P = 0.04). Bars represent median values.
Quantitative Real-Time PCR Analysis. RNA was prepared from ~30 mg of primary tumor tissue, and 100 ng of RNA was used to synthesize cDNA. The TaqMan Gene Expression Assay (Applied Biosystems) was used to evaluate the relative expression levels of mPGES-1, COX-1, and COX-2 mRNA.

The Gene-Expression Database R2. Gene-expression analysis of the impact of mPGES-1 on overall survival in neuroblastoma patients was performed using a publicly available database [R2: microarray analysis and visualization platform (r2.amc.nl)]. In the public Versteeg-88 dataset, mRNA from 88 human neuroblastoma samples are included, 40 of whom are high-risk patients (INSS stage 4).

IHC Analysis of Neuroblastoma Tumor Tissue. Frozen tumor tissue, sectioned at 7 μm and fixed in 2% formaldehyde, was stained. Staining was performed as described earlier (31). All antibodies used in the study are listed in Table S4.


