Regulation by miR181 Family of the Dependence Receptor CDON Tumor Suppressing Activity in Neuroblastoma

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Background

The Sonic Hedgehog (SHH) signaling pathway plays an important role in neural crest cell fate during embryonic development and has been implicated in the progression of multiple cancers that include neuroblastoma, a neural crest cell-derived disease. While most of the SHH signaling is mediated by the well-described canonical pathway leading to the activation of Smoothened and Gli, it has recently been shown that cell-adhesion molecule-related/downregulated by oncogenes (CDON) serves as a receptor for SHH and contributes to SHH-induced signaling. CDON has also been recently described as a dependence receptor, triggering apoptosis in the absence of SHH. This CDON proapoptotic activity has been suggested to constrain tumor progression.

Methods

CDON expression was analyzed by quantitative–reverse transcription–polymerase chain reaction in a panel of 226 neuroblastoma patients and associated with stages, overall survival, and expression of miR181 family members using Kaplan Meier and Pearson correlation methods. Cell death assays were performed in neuroblastoma cell lines and tumor growth was investigated in the chick chorioallantoic model. All statistical tests were two-sided.

Results

CDON expression was inversely associated with neuroblastoma aggressiveness (P < .001). Moreover, re-expression of CDON in neuroblastoma cell lines was associated with apoptosis in vitro and tumor growth inhibition in vivo. We show that CDON expression is regulated by the miR181 miRNA family, whose expression is directly associated with neuroblastoma aggressiveness (survival: high miR181-b 73.2% vs low miR181-b 54.6%; P = .03).

Conclusions

Together, these data support the view that CDON acts as a tumor suppressor in neuroblastomas, and that CDON is tightly regulated by miRNAs.
autocrine expression of the UNC5 ligand netrin-1 and the TrkC ligand NT-3, respectively (7,8). In other human cancers, dependence receptor expression is often reduced or completely lost (9–11). Thus, frequent loss of expression of dependence receptors may be because of epigenetic regulation or loss of heterozygosity (LOH).

In the present study, we investigated the role of cell-adhesion molecule-related/downregulated by oncogenes (CDON), a member of the neural cell adhesion molecule (N-CAM) family in NBs (12,13). SHH canonical signalling includes its interaction with the twelve-transmembrane receptor patched 1 (Ptch). Interaction of SHH morphogen with Ptc prevents Ptc suppressive effect on smoothened (Smo), an orphan seven-transmembrane receptor that initiates a signaling pathway leading to glioma-associated (Gli) transcription factors family activation. However, SHH also appears to interact with other single-transmembrane receptors such as CDON, Gas1, and BOC, positively regulating the hedgehog pathway (14–18). Recently, we described a new role of dependence receptor (19) for CDON: upon enforced expression, CDON triggers apoptosis in various cancer cell lines, and this proapoptotic activity is blocked by the addition of SHH in the medium (20). As for other dependence receptors, CDON’s proapoptotic activity requires caspase cleavage of its intracellular domain, leading to the exposure of a proapoptotic domain able to recruit and activate the apical caspase-9. We proposed that this proapoptotic activity is a safeguard mechanism to limit cancer progression (20).

In the present study, we show that CDON acts as a tumor suppressor gene whose expression is tightly controlled by miRNAs in NBs. miRNAs are small non-coding RNA molecules (20–22 nucleotides) found in plants and animals, which function in the transcriptional and post-transcriptional regulation of gene expression. This mechanism potentially explains the loss of CDON expression observed in high-grade NBs.

**Methods**

A complete description of the methods, including in vitro cell death and proliferation experiments, quantitative reverse transcription–polymerase chain reaction (RT-PCR), assessment of mRNA expression, immunoblots, soft agar assays, and TUNEL staining, is presented in the Supplementary Materials (available online).

**Ethics, Human Tumor Samples, and Biological Annotations**

The use of all patient tissue specimens was carried out according to French laws on the protection of biomedical research subjects. Human neuroblasticoma samples were collected from a retrospective cohort of 226 patients staged according to the International Neuroblastoma Staging System (21). Tissue samples were collected between 1987 and 2009 at the Centre Leon Bérard, Lyon (n = 12), at the Institut Gustave Roussy, Villejuif (n = 214), and Curie institute, Paris (n = 20). Following patient consent, primary tumor tissues were obtained, either by biopsy or after surgery, and were immediately frozen before being stored in liquid nitrogen until RNA/DNA extraction. For each institution, the nature of the study was approved by the respective institutional board before releasing samples and data (Centre de Ressource Biologique, CRB Copil).

**Cell Lines, Transfection Procedures, Reagents, Plasmid Constructs, and siRNA**

Cell lines were cultured and transfected as described in the Supplementary Materials (available online). CDON 3′-UTR fragments were PCR-amplified and cloned in the bicistronic pmIR-GLO vector. Point mutations for rs3737336 SNP were created using the QuikChange site directed mutagenesis strategy (Stratagene, La Jolla, CA).

**Chick Chorioallantoic Model for Neuroblastoma Progression**

Chick chorioallantoic assays were carried out according to French laws and regulations and institutional guidelines as previously described (22). SH-EP cells were transfected with plasmids 24 hours after plating, and harvested 24 hours posttransfection. SH-EP transfected cells (5 × 10^6) were suspended in 50 μL phosphate-buffered saline and 50 μL of matrigel. These cells were seeded on 10-day-old (day10) chick chorioallantoic membrane (CAM) previously locally injured with a cotton swab. On day 17, tumors were dissected and the area was measured with ImageJ software (open source, National Institutes of Health, Bethesda, MD).

**Statistical Analysis**

The quantitative RT-PCR (Q-RT-PCR) data were analyzed using GraphPad-Prism (GraphPad Software, San Diego, CA) or R software (Free software foundation, University of Auckland, New Zealand) to generate scatter plots, and unpaired Student’s t test was used to compare the different NB groups. Relative expression of each gene was calculated according to the comparative 2−ΔΔCt quantification method where ΔΔCt=Ct(sample)−Ct(normaliser) and ΔCt=ΔCt(sample)−ΔCt(calibrator). T tests were performed after this transformation of the data.

To test mRNA expression, cohorts were separated based on hsa-miR181-a, -b, -c, and -d median expression. Survival curves were generated by the Kaplan–Meier method on GraphPad software. Correlations of gene expression were determined using Pearson coefficient or R^2 with scatter plots of gene expression. Data were analyzed with a Mantel-Cox test. n indicates the number of repeats. All statistical tests were two-sided, and a P value of less than .05 was considered statistically significant.

**Results**

**Analysis of CDON Expression in Neuroblastoma**

NB outcome is highly associated with tumor grade according to the INSS classification. An 85.3% 10-year survival was observed in the low-risk group, whereas the high-risk group presents aggressive tumors leading to more frequent death, with an overall survival of 29.3% after 10 years (Mantel-Cox test on high- vs low-risk NBs, P ≤ .001) (Supplementary Figure 1A, available online). To assay the implication of the CDON receptor in NB, CDON gene expression was analyzed by Q-RT-PCR in a panel of 121 NBs. While CDON was detected at a relatively high level in stages 1 or 2 NBs, which are localized NBs with low aggressiveness, CDON expression decreased with higher-staged, more aggressive tumors. Thus, Stage 4 NBs, which are highly aggressive and metastatic tumors with poor outcome, displayed the lowest level of CDON (unpaired Student’s t test on stage 1 vs stage 4, P ≤ .001) (Figure 1A). Interestingly,
the stage 4S NB, which specifically affects neonates and encompasses highly metastatic tumors that nonetheless often spontaneously regress, displayed a level of CDON similar to that observed in stage 1–2 NBs (unpaired Student’s t test on stage 4 vs stage 4S, \(P = .001\)) (Figure 1A). As shown in Figure 1B, a similar expression pattern was seen on tumor sections at the protein level. Thus elevated CDON expression is strongly associated with an overall positive prognosis in the 107-patient cohort (Figure 1C). The overall survival at 10 years in all NBs tested was 74.3% for high CDON-expressing tumors but only 33.3% for low CDON-expressing tumors (Mantel-Cox test on high vs low CDON expression groups, \(P \leq .001\)) (Figure 1C). When considering only highly aggressive stage 4 NBs (n = 70), the overall survival at 10 years was 46.8% for high CDON-expressing tumors but only 23.8% for low CDON-expressing tumors (Mantel-Cox test on high vs low CDON expression groups in stage 4 NBs, \(P = .05\)) (Figure 1D).

Effects of CDON on Neuroblastoma Cell Death

We therefore hypothesized that CDON expression represents a constraint for tumor progression in NBs. In agreement with the dependence receptor paradigm, some NB cell lines should have selected low CDON expression to survive. Expression of CDON was investigated in a panel of NB cell lines by Q-RT-PCR, and, as shown in Supplementary Figure 1B (available online), half of the tested cell lines showed no expression, or a barely detectable level of expression, of CDON. We further investigated the importance of CDON in SH-SY-5Y and SH-EP cells, which show a weak or nonexpression of CDON. We first assessed the effect of CDON overexpression in these two NB cell lines. CDON transient overexpression was found to be associated with an increase in caspase-3 activity, a hallmark of apoptosis (Figure 2A). Moreover, such a proapoptotic effect was caspase-dependent, because the addition of the general caspase inhibitor z-VAD-fmk inhibited CDON-induced cell death. We therefore investigated whether, as expected from the dependence receptor paradigm, addition of the SHH ligand could prevent CDON-induced cell death. As shown in Figure 2A, cell death resulting from CDON expression is inhibited by addition of exogenous recombinant SHH. In addition, CDON expression resulted in decreased anchorage-independent growth (Figure 2B). This effect was prevented by the addition of recombinant SHH. However, the activation of the canonical pathway by a smoothened (Smo) agonist, SAG, failed to interfere with CDON inhibitory activity, thus supporting the view that the canonical SHH pathway is not implicated in these effects (Figure 2B, right panels). Because these experiments were based on enforced expression of CDON in NB cell lines that do not express CDON, we next searched for a loss-of-function approach by which to analyze the endogenous
proapoptotic activity of CDON. Based on the analogous function of other dependence receptors, we assessed whether in NBs with CDON expression, SHH secretion constitutively blocks CDON proapoptotic activity. IMR32 cells were therefore treated with the SHH blocking antibody that is known to interact with SHH in the region where CDON binds to SHH (23). The SHH blocking antibody treatment was associated with cell death induction and this effect was partly blocked when CDON was silenced by a siRNA approach (Supplementary Figure 1C, available online).

To analyze the tumor suppressive activity of CDON in vivo, SH-EP cells with or without enforced CDON expression were xenografted to the chorioallantoic membrane (CAM) of ten-day-old chick embryos (Figure 2C). The CAM of chicken embryos is a well-described model to study NB tumor progression (22). Seventeen-day-old chick embryos were analyzed for primary tumors. Recombinant Sonic Hedgehog (SHH) was added in the cell culture medium. The general caspase inhibitor z-VAD-fmk was used to block caspase-dependent cell death. Error bars indicate s.e.m. Statistical treatment of the data was performed using the Mann-Whitney test (*P < .05).

Figure 2. CDON prevents tumor progression in neuroblastoma models. A) Cell death induction in SH-EP and SH-SY5Y cells was quantified by caspase-3 activity assay after transfection with mock or cell-adhesion molecule-related/downregulated by oncogenes (CDON) expression vectors. Recombinant Sonic Hedgehog (SHH) was added in the cell culture medium. The general caspase inhibitor z-VAD-fmk was used to block caspase-dependent cell death. Error bars indicate s.e.m. Statistical treatment of the data was performed using the Mann-Whitney test (*P < .05). B) Soft agar assay for colony formation was performed on cells 14 days after transfection with CDON encoding construct alone or after addition of recombinant SHH, Smoothened Agonist (SAG) or DMSO (SAG’s diluent). The mean number of clones per dish and per condition is presented as means of at least three independent assays. Error bars indicate SD. Statistical treatment of the data was performed using a two-sided Mann-Whitney test compared with mock-transfected condition (**P < .01, ***P < .001, n = 3). C) Schematic representation of the experimental chick chorioallantoic membrane (CAM) model is presented. SH-EP cells, transiently transfected with various plasmids, were xenografted on CAM at day 10. Tumors were harvested on day 17, measured, and sectioned for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. D) Representative images of SH-EP formed tumors described in C were presented. Scale bar represents 2 mm. E) Quantitative analysis showing the size of the respective primary tumors described in D (*P < .05, two-sided Mann-Whitney test). F) Representative images of TUNEL-positive staining in sections performed on the respective primary tumors described in D are presented. Scale bar represents 50 μm. G) TUNEL-positive cells described in F are quantified. (**P < .01, two-sided Mann-Whitney test).

Effects of the hsa-miR-181 Family on CDON Expression
Because CDON appears as a key regulator for NB progression, we investigated the mechanisms for CDON expression regulation in NBs. Interestingly, the CDON gene resides at human chromosome 11q24.2, a region often included in the 11q23-24 deletion detected at high frequency in NBs (24,25), which may potentially explain a reduction of CDON level in aggressive NBs. However, based on the analysis of 19 NBs in which CDON mRNA analysis could be associated with 11q status analysis, we failed to observe any association between CDON mRNA expression and 11q status (Figure 3A), suggesting that CDON downregulation in aggressive NBs is not simply because of a loss of the chromosome 11q24.2 region.

We thus hypothesized that CDON may be regulated at the posttranscriptional level by miRNA regulation. miRNAs are capable of blocking translation and/or inducing target mRNA degradation. We found via an in silico screen that the CDON 3’UTR could putatively be bound by miR181 miRNAs (Figure 3B). Moreover, analysis softwares like TargetScan and miRBase predict an elevated hybridization score and a highly conserved binding site among organism evolution on the CDON 3’UTR (Supplementary Figure 2A, available online).

miR181-a and -b, as revealed by the microarray analysis of 15 patients, are both upregulated in high-risk NBs (P = .01 and .004,
respectively) (Figure 3C) (26), supporting an inverse association between CDON expression and miR181. In the same analysis, miR181-c and -d expression were not affected (Supplementary Figure 2B, available online).

To analyze the effect of miR181 family on CDON gene regulation, CDON 3’-UTR was fused with the firefly luciferase gene in the pmiR-GLO vector (Figure 3D). As shown in Figure 3D, enforced expression of mimic forms of miR181-a, -b, -c, and -d induced a decrease of the luciferase activity of the reporter construct (two-sided Mann-Whitney test, miR-C vs miR181-a, -b, -c, -d, *P < .05). Moreover, transfection of miR181-a and -b mimics in the IGR-N-91 neuroblastoma cell line induced a decrease of CDON at the RNA level, suggesting a destabilization of the transcript in this cell line (miR-C vs miR181-a, -b P = .03 and P = .01, respectively) (Figure 3E). Additionally, as revealed by immunoblots, CDON protein level decreased upon transfection of each miR181 family member (Figure 3F).

An association between miR181 upregulation and poor prognosis has been shown among breast cancer patients (27). Our findings confirmed this in silico data obtained by microarray analysis (Figure 3C), by quantification of all isoforms of the miR181 family by Q-RT-PCR in a cohort of 226 NB patients (Figure 4, A and B). Stages were separated from the whole cohort on the basis of metastatic progression: stage 1, 2, and 3 vs stage 4. Stage 4S was included in stage 1, 2, 3 groups because the prognosis of these patients is usually comparable. Figure 4, B and C, shows that the expression of miR181-a and -b was increased in aggressive stage 4 in comparison with less aggressive NB forms (P = .04 and P = .03, respectively). miR181-a and -b are encoded by two loci, forming two gene clusters on chromosomes 1 and 9, and coexpression in human NBs was highly correlated (Pearson coefficient or R2 close to 1, with 1 indicating perfect coexpression) (Figure 4D; Supplementary Figure 3, D and E, available online). Differences in expression of miR181-c and -d were not observed (Figure 4D; Supplementary Figure 3, A and B, available online).

Expression of miR181-a and miR181-b, which appear more specifically to induce CDON mRNA degradation (Figure 3E), showed a substantial correlation with patient prognosis. As shown in Figure 4, E and F, the overall survival at 10 years is 52.8% vs 71.4%; 54.6% vs 73.2% for high miR181-a and -b NBs compared with low miR181-a and -b NBs (Mantel-Cox test on high vs low miR-181-a and -b expression groups, P = .06 and P = .03, respectively). miR181-c and miR181-d expression showed no association with NB prognosis (Supplementary Figure 3, F and G, available online).

Figure 3. hsa-miR181 family regulates CDON expression in neuroblastoma. A) Cell-adhesion molecule-related/downregulated by oncogenes (CDON) mRNA expression is analyzed by quantitative–reverse transcription–polymerase chain reaction (Q-RT-PCR) using hypoxanthine-guanine phosphoribosyltransferase HPRT as a housekeeping gene in a panel of 19 stage 4 neuroblastomas (NBs) classified according to chromosome 11 status: normal chromosome 11 (black), heterozygous loss of 11q chromosome (light gray), heterozygous loss of whole chromosome 11 (brown). B) Prediction of hybridization of hsa-miR181-a, -b, -c, and -d is presented with CDON 3’-untranslated region (UTR) (adapted from www.targetscan.org). C) Fifteen NB patients’ miRNAs samples were submitted to microarray analysis. Patients were classified by prognosis into high- vs low-risk NBs. Expression of 851 human miRNAs was analyzed (26). D) CDON mRNA 3’-UTR cloned on a bicistronic pmiR-Glo luciferase assay and were transfected in NIH-3T3 cells with/without miRNA mimics. After 48 hours, the luciferase activities were measured. Firefly luciferase activity is normalized to the renilla luciferase activity (n = 4, *P < .05, two-sided Mann-Whitney test). E) Quantification of CDON mRNA is realized by Q-RT-PCR after transfection of miRNA mimic in neuroblastoma cell lines (two-sided Student’s t test). F) Endogenous level of CDON is decreased in hsa-miR181 family transfected cells. Cells lysates were collected, and samples were analyzed by immunoblotting using antibodies that recognize CDON polypeptides.
Our results also confirm previous microarray studies, which showed an increase of miR181-a and -b in aggressive NBs (28). The array data also suggested a direct role of MYCN oncogene amplification in miR181-a and -b regulation (28). In Supplementary Figure 4, A and B, miR181-a and -b were more highly expressed in MYCN amplified cell lines as compared with SH-SY-5Y and SK-N-AS, two MYCN nonamplified cell lines. However, we failed to observe any relation between miR181-a and -b expression and MYCN amplification in the patient cohort, suggesting a more complex regulation (Supplementary Figure 4C, available online).

Analysis of Rs3737336 SNP as a Putative Diagnosis Marker in NBs

A single-nucleotide polymorphism (SNP), referenced as rs3737336, was detected in the CDON 3’UTR at the predicted miR181 family binding site (Figure 5A). We hypothesized that this SNP could act as an inactivating mutation for miR181 regulation of CDON expression. As shown in Figure 5, B and C, forced expression of miR181-a, -b, -c, and -d mimics failed to decrease luciferase activity of a luciferase reporter construct containing the rs3737336, supporting an inhibitory role of rs3737336 in miR181 binding.

Moreover, we analyzed the presence of the rs3737336 SNP in NBs. This SNP had already been described to modify patient response in prostate cancer hormone therapy (29). CDON expression was quantified by Q-RT-PCR in a fraction of the 91-NB cohort, in which tumoral DNA and miRNA expression could be tested simultaneously (Figure 5D, left panel). Patient genotyping was performed by direct tumor DNA sequencing and associated with CDON mRNA expression. We compared the tumors that were homozygous for the functional binding site (A/A) with those that were either heterozygous (A/G) or homozygous (G/G) for the nonfunctional sequence, because we could not discard a possible bias in the analysis since A/A and G/G patients could actually be 11q23-24 deleted. As shown in Figure 5D, patients bearing a rs3737336 (A/A) SNP present a lower expression of CDON than the ones with A/G-G/G SNP. Similar results were obtained with a second independent NB cohort (unpaired Student’s t test on A/A vs AG group, P = .05 and P = .005) (Figure 5D, lower panel). To address the implication of rs3737336 in NBs, we next tested the prognostic value of the polymorphism (Mantel-Cox test on A/A vs A/G group, P = .08).

Discussion

In the present study, we show that CDON expression is reduced in aggressive NB stages. This low CDON expression associates with poor prognosis for patient survival. We also describe possible mechanisms for CDON expression regulation in NBs. Part of the relatively low level of CDON may be attributed to the 11q23-24 deletion detected at

Figure 4. hsa-miR181-a and -b are upregulated in bad prognosis neuroblastoma, and their levels correlate with patient outcome. A) hsa-miR181-a, -b, -c, -d expression were detected by quantitative–reverse transcription–polymerase chain reaction (Q-RT-PCR) on a panel of neuroblastoma (NB). The ubiquitously expressed Small Nucleolar RNA C/D Box 44 (RNU44) was used as an internal control for neuroblastoma. B-C) hsa-miR181-a and -b expression were quantified by Q-RT-PCR in a panel of human stages 1–2, 3, 4 and 4S NBs (n = 219 for hsa-miR181-a; n = 216 for hsa-miR181-b). Right panel represents the statistical analysis.
high frequency in NBs (24,25). However, we propose here that CDON mRNA and protein could be regulated by miRNAs. Compatible with this hypothesis, we show that miR181 family members directly affect CDON mRNA/protein expression in some NBs cell lines. We describe that hsa-miR181-a and -b expression are upregulated in aggressive NBs, and that high hsa-miR181-a and -b are associated with poor prognosis. Interestingly, miR181 has already been shown to be associated with poor prognosis in CRCs (30). More specifically, miR181-a expression has been proposed as a prognostic marker for patients with CRCs treated with epidermal growth factor receptor inhibitor (31).

Interestingly, the presence of the rs3737336 polymorphism in the CDON 3′-UTR may interfere with the miR181 family binding site. We demonstrate that this SNP inhibits miR181 binding and associates with poor patient prognosis, strengthening the importance of the negative regulation of CDON in NBs.

The regulation of CDON by miRNAs in vitro and the demonstration here that CDON is a tumor suppressor in NBs raise the question of whether other dependence receptors could also be regulated by miRNAs in neoplasms. It is indeed known that dependence receptors such as DCC or Trk-C are downregulated in multiple cancers, allowing tumor cell survival (10,32,33), but little is known about whether miRNAs are implicated in these downregulations. A specific isoform of Trk-C has been shown to be regulated by miRNA-9, 125, and 125b in NBs, but the functional effect described failed to match with the known positive prognostic value of high Trk-C expression in NBs (34).

The current study has nevertheless some limitations. First, at this stage we cannot exclude that the rs3737336 polymorphism creates a new binding site for a new miRNA in CDON 3′-UTR, modifying miR181 family binding. This will need to be investigated further. Another relative limitation of the study is the fact that, although we have shown a direct regulation of CDON expression by miR181 in cell culture, we can only suspect a link between miR181 and CDON expression in patients. However, the description here that CDON re-expression is sufficient to block NB cell line growth, both in vitro and in vivo, probably via its dependence receptor function and its strong regulation by hsa-miR181, supports the view that inhibition of proapoptotic proteins by miRNAs is an important feature for NB development. Along this line, it is worth noting that the miR181 family appears to regulate Bim, a classic BH3-only Bcl protein, in breast cancer cells, with an impact on promoting metastasis (27). Whether miR181 also regulates the tumor suppressive function of CDON in other cancers will now be of interest to investigate.

References


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