

# Different subcellular localization of ALCAM molecules in neuroblastoma: Association with relapse

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**Abstract.** *Background:* The Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166), involved in nervous system development, has been linked to tumor progression and metastasis in several tumors. No information is available on ALCAM expression in neuroblastoma, a childhood neoplasia originating from the sympathetic nervous system.

*Methods:* ALCAM expression was analysed by immunofluorescence and immunohistochemistry on differentiated neuroblastoma cell lines and on archival specimens of stroma-poor, not *MYCN* amplified, resectable neuroblastoma tumors, respectively.

*Results:* ALCAM is variously expressed in neuroblastoma cell lines, is shed by metalloproteases and is cleaved by ADAM17/TACE *in vitro*. ALCAM is expressed in neuroblastoma primary tumors with diverse patterns of subcellular localization and is highly expressed in the neuropil area in a subgroup of cases. Tumor specimens showing high expression of ALCAM at the membrane of the neuroblast body or low levels in the neuropil area are associated with relapse ( $P = 0.044$  and  $P < 0.0001$ , respectively). *In vitro* differentiated neuroblastoma cells show strong ALCAM expression on neurites, suggesting that ALCAM expression in the neuropil is related to a differentiated phenotype.

*Conclusion:* Assessment of ALCAM localization by immunohistochemistry may help to identify patients who, in the absence of negative prognostic factors, are at risk of relapse and require a more careful follow-up.

**Keywords:** Neuroblastoma, ALCAM/CD166, immunohistochemistry, prognosis

## 1. Introduction

Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166) is a member of the Immunoglobulin gene superfamily, which mediates cell–cell clustering through homophilic (ALCAM–ALCAM) and heterophilic (ALCAM–CD6) interactions (reviewed in Swart [30]). In adult tissues ALCAM expression is limited to subsets of cells, whereas in several human tumors, including melanoma, prostate, breast, bladder and colorectal cancer, alterations in expression of ALCAM have been reported (reviewed in Ofori-

Acquah and King [17]). By mean of an anti-ALCAM recombinant single chain antibody (scFv I/F8), we previously showed that ALCAM is expressed at the surface of epithelial ovarian cancer (EOC) cells, can be internalized following soluble ligand engagement [21] and is released in a soluble form (sALCAM) by a disintegrin and metalloprotease (ADAM)17/TACE-dependent mechanism [24]. ALCAM shedding mediated by ADAM17/TACE is relevant to EOC cell motility, and the decreased membrane expression of ALCAM is a marker of poorer outcome in EOC patients [15]. ALCAM role in tumor progression and metastasis has been well documented in other tumors. In melanoma, high levels of ALCAM membrane expression correlate with the vertical growth phase of tumor progression [33]. Transfection of a cDNA encoding for a dominant negative, amino-terminally

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truncated, ALCAM form increased spontaneous lung metastasis in a transplantable melanoma model, indicating that suppression of surface ALCAM adhesive functions is required to mobilize cells from primary tumors (reviewed in Swart et al. [31]). Thus the dynamic control of ALCAM at the cell surface seems relevant to the progression of different tumor types.

Given the documented role of ALCAM in central nervous system development and its pathfinding activity in neurite extension [19,35], we investigated ALCAM expression in human neuroblastoma (NB) cell lines and evaluated whether different ALCAM expression in primary tumors was associated with different outcomes. In addition, recent data in a murine model of NB indicated that ALCAM represents an antigenic target for immune recognition on mouse NB cells [36]. NB is a rare sympathetic nervous system neoplasia with a broad spectrum of clinical presentations, varying from aggressive disease (stage 4) to spontaneous maturation and even regression (stage 4S). Prognosis depends on age, stage, histology and genetic features [4,27]. While prognosis for stage 4 patients is grim, that for patients with resectable NB is good [9]. However, approximately 10% of the latter develop local or metastatic relapse that may be fatal. In addition to *MYCN* amplification [25], few prognostic factors have been described so far for these patients [8,20,26,28,29], including the histopathological features of the tumors [16]. Since these factors identify only subsets of patients with localized resectable NB at risk of relapse, the search for novel markers is warranted.

## 2. Materials and methods

### 2.1. Cells, reagents and antibodies

Human NB cell lines GI-CA-N, GI-LI-N and GI-ME-N were established at the Laboratory of Oncology, Gaslini Institute, Genoa, Italy. IMR-32 and SK-N-SH were from ATCC (Rockville, MD, USA); SK-N-AS, SK-N-F1, SH-SY-5Y and IMR-5 were from ECACC (Genoa, Italy). LAN-1 and LAN-5 were a kind gift from Dr. R. Seeger (Los Angeles, CA, USA). SK-N-BE and ACN were kindly provided by Dr. J. Biedler (New York, NY, USA) and Dr. S. Carrel (Lausanne, Switzerland), respectively. All cell lines were grown in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine and penicillin-streptomycin (100 µg/ml) (BioWhittaker Cambrex, Verviers, Belgium).

Chemicals were from Sigma Chemical Co. (St. Louis, MO, USA), as well as the anti- $\beta$  actin and anti-FLAG (clone M2) mAb. Anti-ALCAM scFv I/F8 and anti-NIP scFv have been described [21]. Monoclonal anti-ALCAM antibody MOG/07 was purchased from NovoCastra Laboratories (Newcastle upon Tyne, UK). Anti-ADAM17/TACE rabbit polyclonal antibody was from Abcam (Cambridge, UK). FITC-, Alexa488- or HRPO-conjugated secondary antibodies were from Caltag/Invitrogen (Paisley, UK).

### 2.2. Immunofluorescence

Immunofluorescence was performed incubating  $10^5$  viable cells with 5 µg/ml scFv I/F8 (or anti-NIP scFv as negative control) plus 2 µg/ml anti-FLAG mAb for 40 min on ice. As secondary reagent, FITC conjugated (Fab)<sub>2</sub> goat anti-mouse Ig was used. Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA, USA) flow cytometer.

Immunofluorescence on cells grown in chamber slides (BD Biosciences, Bedford, MA, USA) was performed as above, using Alexa488-conjugated (Fab)<sub>2</sub> goat anti-mouse Ig as secondary reagent. Cells were then fixed (1% PFA in PBS), permeabilized (0.2% Triton-X-100 in PBS) and cell nuclei were counterstained with propidium iodide [21]. Mowiol-mounted slides were analysed by confocal fluorescence microscopy using an Olympus (Olympus Optical, Tokyo, Japan) laser-scanning microscope FV500 equipped with an Olympus IX81 inverted microscope, as described elsewhere [21].

### 2.3. Immunoprecipitation and Western blot analysis

Immunoprecipitation of ALCAM and sALCAM molecules from cell lysates and conditioned media was performed as described [21, 24] and precipitated molecules were resolved by 11% acrylamide SDS-PAGE. ALCAM molecules were revealed by Western blotting with MOG/07 mAb, according to standard procedures, and visualized by chemiluminescence (ECL, GE Healthcare).

The ability of human recombinant ADAM17/TACE (R&D Systems, Minneapolis, MN, USA) to cleave full-length ALCAM was studied by *in vitro* treatment of ALCAM molecules immunoprecipitated by scFv I/F8-sepharose from SK-N-BE cell lysates. Sepharose-bound immunoprecipitated molecules were moved to two centrifugal concentrators (cut off 3,000; Vivascience/SIGMA), thoroughly washed with water and

then with digestion buffer (25 mM Tris-HCl pH 9.0, 2.5  $\mu$ M ZnCl<sub>2</sub>, 0.005% Brij 97). ADAM17/TACE (1  $\mu$ g/sample) or digestion buffer alone was added and incubation carried out overnight at 37°C. Digestion products were analyzed by Western blot as above.

#### 2.4. Cell treatments and ELISA

To assess the shedding of the ALCAM molecule, subconfluent cells were cultured in 24 wells plates in medium 0.1% FCS plus 10  $\mu$ M CGS27023A (CGS) metalloprotease inhibitor [23] or the equivalent amount of its DMSO solvent. If required, after 30 min at 37°C, 200  $\mu$ M pervanadate (PV) [6] or, as controls, sodium orthovanadate (OV) or H<sub>2</sub>O<sub>2</sub> was added and incubation prolonged for 30 min. Conditioned media were then collected and tested for sALCAM by an ELISA assay (DuoSet ELISA Development kit, R&D System) in triplicates. Data were expressed as the mean  $\pm$  SD, and were analysed using a two-tailed Student's *t*-test.

For differentiation of the NB cell lines, cells were seeded in tissue culture slides (BD Biosciences) and allowed to adhere for at least 24 h. Culture media were then replaced by fresh medium containing 10  $\mu$ M all-trans retinoic acid (ATRA, Sigma) and cells were incubated for the indicated times, with daily changes of the ATRA-containing medium.

#### 2.5. Study subjects

Among the Italian patients with localized NB, enrolled in the LNSG1 or 94.01 protocols [10,16], 23 patients with resectable tumors, 13 stage 1 and 11 stage 2, as assessed according to INSS criteria [3], and without *MYCN* amplification were selected for a case/control study. Eleven of them experienced relapse, either local (*N* = 6) or metastatic (*N* = 5), while the remaining 12 were in complete remission. Three out of the 11 relapsing patients died, 2 for local progression and 1 of metastatic disease. The median follow-up was of 4.8 years (range 0.96–8.8 years). The two groups of patients did not show any significant difference as to age, gender and stage. Moreover, none of them presented known negative prognostic factors [8,16,20,26,28,29]. All tumors were schwannian stroma poor with different differentiation grade and different mitotic kar-

ioretic index (MKI). The use of tissue blocks and patient records was approved by the Institutional Review Board of the Gaslini Institute.

#### 2.6. Immunohistochemistry

The 23 paraffin-embedded neuroblastoma tumors, stored at the Department of Pathology, Gaslini Institute (Genoa, Italy), were analyzed by a three steps indirect immunoperoxidase technique, as previously described [12]. Anti-ALCAM (clone MOG/07, NovoCastra) and a peroxidase-labelled dextran polymer conjugated anti-mouse antibody (Dako) were used in the first and second step, respectively. Slides were counterstained with Mayer's hematoxylin.

#### 2.7. Score and grading analysis

The immunohistochemical results were classified using two different systems. With one system the staining was qualitatively scored as described for Her2/neu expression (Dako, Copenhagen, Denmark) [11]. Precisely, according to the Herceptest, samples were scored 0 in absence of staining; 1 in the presence of weak and partial staining in more than 10% of cells and neuropil; 2 when moderate staining was detected in more than 10% of cells and neuropil and 3 when intense staining occurred in more than 10% of cells and neuropil.

With the second system the staining was graded semi-quantitatively, as described previously [12]. Grading, independently applied to membrane and neuropil area was as following: +/- with 10–25% positive tumor cell structures; + with 25–50% positive tumor cell structures; ++ with 50–75% positive tumor cell structures and +++ with 75–100% positive tumor cell structures.

#### 2.8. Survival and statistical analysis

Clinical data of NB patients were retrieved from the Italian Neuroblastoma Registry (INBR) [7]. Survival curves were constructed by using the Kaplan-Meier method and the generalized Wilcoxon log-rank test was used to compare the curves. A *P*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using Prism 3 (GraphPad Software, San Diego, CA, USA).

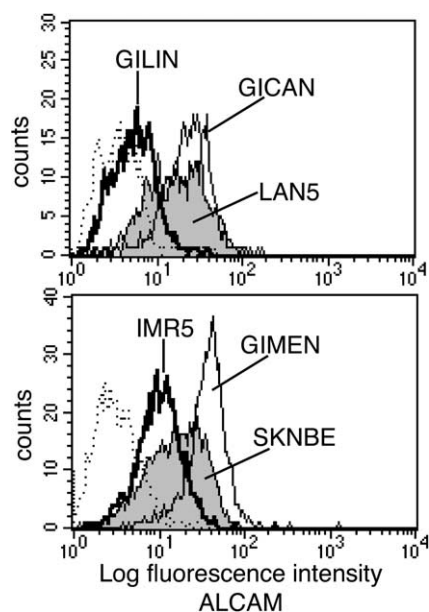


Fig. 1. Cell surface expression of the ALCAM molecule by human neuroblastoma cell lines. Immunofluorescence analysis of ALCAM surface expression by anti-ALCAM scFv I/F8 staining and flow cytometry. Dotted lines represent background fluorescence obtained with an irrelevant scFv.

### 3. Results

#### 3.1. NB cell lines express ALCAM and constitutively release ALCAM ectodomain in a soluble form.

A panel of 13 human neuroblastoma cell lines was analysed for cell surface ALCAM expression by flow cytometry. As shown in Fig. 1, ALCAM was expressed at various levels in different cell lines, ranging from very low (GI-LI-N) to high levels (GI-ME-N). No relationship was found between cell surface ALCAM and *MYCN* amplification or chromosome 1p deletion status [32] (Table 1). As proteolytic release of ALCAM may influence its membrane expression, we evaluated whether ALCAM was shed by NB cells, as described for ovarian carcinoma cells [24]. The metalloprotease ADAM17/TACE, which is involved in ALCAM proteolytic cleavage, was indeed expressed in NB cells as detected by WB analysis (Fig. 2A). In addition, immunoprecipitation with the anti-ALCAM I/F8 scFv, followed by western blot analysis, showed the presence of two soluble ALCAM (sALCAM) forms of approximately 95 and 65 kDa in conditioned media from NB cells (Fig. 2B). Moreover, to evaluate if sALCAM was generated by metalloprotease activity, we treated GI-CA-N cells with pervanadate (PV,

Table 1  
Cell surface ALCAM expression by human NB cell lines

Cell line	Mean fluorescence intensity*	<i>MYCN</i> amplification	Chromosome 1p deletion
GI-ME-N	6.6	no	yes
GI-CA-N	6.5	no	no
SH-SY-5Y	5.7	no	no
LAN-5	4.9	yes	yes
SK-N-BE	4.5	yes	yes
SK-N-AS	4.4	yes	yes
LAN-1	4.0	yes	yes
SK-N-F1	3.9	no	no
SK-N-SH	3.4	no	no
IMR-5	1.7	yes	no
ACN	0.9	no	no
IMR-32	0.8	yes	yes
GI-LI-N	0.4	yes	yes
A2774 (ovary)	8.5		

Note: \*Mean fluorescence intensity is calculated as ratio between mean of sample after subtraction of background fluorescence and mean of background fluorescence.

a known activator of metalloproteases) in the presence of the ADAMs inhibitor CGS27023A (CGS). As shown in Fig. 2C, a strong inhibition of both spontaneous and PV-inducible ALCAM shedding was observed, as assessed by ELISA. Altogether these data suggest that dynamic control of surface ALCAM expression is detectable in neuroblastoma. Although previous evidences indicated that ADAM17/TACE is involved in ALCAM shedding, no formal proof that this enzyme directly cleaves ALCAM was provided. We therefore challenged full-length ALCAM, immunoprecipitated from NB cell lysates, with recombinant human ADAM17/TACE and searched for cleavage products by Western blot. As shown in Fig. 2D, the 65 kDa sALCAM form was clearly generated by ADAM17/TACE activity, thus providing the first direct evidence of ALCAM cleavage by this metalloprotease.

#### 3.2. *In vitro* differentiated NB cells show strong ALCAM expression on neurites

We further analysed the pattern of ALCAM distribution in human NB cells following all-trans retinoic acid (ATRA)-induced differentiation of the SK-N-BE and SH-SY-5Y cell lines. First of all, no increase of soluble ALCAM, regarded as a marker of ADAM17/TACE activity, was detected in retinoic-acid-treated NB cell

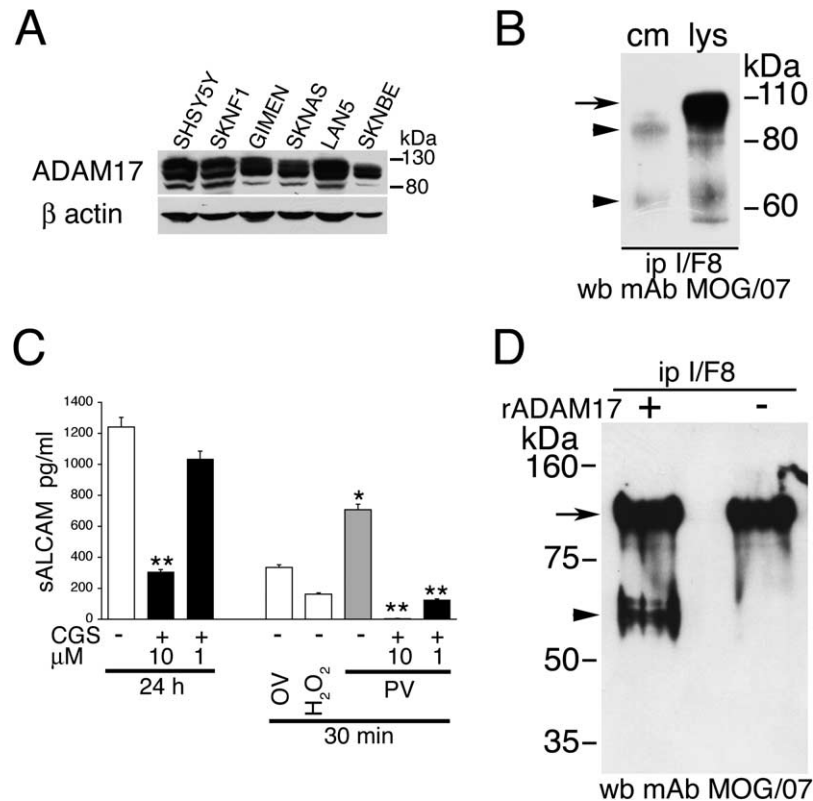


Fig. 2. ALCAM shedding by neuroblastoma cell lines. (A) Neuroblastoma cell lines express ADAM17/TACE, as assessed by Western blot analysis of cell lysates (50  $\mu$ g/lane). Analysis of  $\beta$ -actin was used as loading control. Numbers 130 and 80 (kDa) indicate the glycosylated ADAM17/TACE zymogen and its active enzyme form, respectively. (B) Western blot analysis of the ALCAM molecules immunoprecipitated by I/F8 scFv from GI-CA-N cells lysate (lys) and conditioned media (cm). Arrowheads: sALCAM; arrows: membrane ALCAM. (C) Shedding of sALCAM is metalloprotease-dependent. ELISA detection of sALCAM released by GI-CA-N NB cells in the presence of the ADAM17/TACE inhibitor CGS. Constitutive (24 h) and pervanadate (PV)-induced release are shown. PV was freshly prepared for each experiment by mixing sodium orthovanadate (OV) and H<sub>2</sub>O<sub>2</sub> and was used within 20 min of preparation. OV and H<sub>2</sub>O<sub>2</sub> were used as controls. Mean  $\pm$  SD of two experiments. \* $P$  < 0.05 vs treatment with OV or H<sub>2</sub>O<sub>2</sub>; \*\* $P$  < 0.005 vs no CGS sample. (D) ADAM17/TACE directly cleaves full-length ALCAM. Cellular ALCAM molecules were immunoprecipitated from SK-N-BE cell lysates by sepharose-bound I/F8 scFv, challenged with either recombinant ADAM17/TACE or buffer alone and analysed by Western blot.

cultures (Fig. 3A). Moreover, no significant changes in ADAM17/TACE protein expression were induced by ATRA treatment in both SH-SY-5Y (Fig. 3B) and SK-N-BE NB cell lines (not shown). Secondly, confocal microscopy (Fig. 4) showed that after ATRA treatment, surface ALCAM expression was evident on the neurites and dendrites of differentiated NB cells, becoming particularly strong on the neurites after 7 days of treatment (Fig. 4Ad, Be). It is noteworthy that in optical sections the density of surface ALCAM expression appeared higher on the neurites than on the cell body, especially in SK-N-BE cells (Fig. 4B). The acquisition of this ATRA-induced phenotype was independent on ADAM17/TACE activity, as it was not influenced by treatment with pharmacological blockers of ADAM-17/TACE activity (not shown). Taken

together, *in vitro* data indicate that in differentiating NB cells ALCAM redistributes to the neurites and that ADAM17/TACE is apparently not involved in this process.

### 3.3. ALCAM subcellular localization in NB tumor samples

We recently described that the subcellular localization of the ALCAM molecule correlates with a worse prognosis in EOC patients [15]. The observation that different NB cell lines, that derive from high risk tumors, exhibited various levels of surface ALCAM expression and that ATRA-treated NB cells, regarded as an *in vitro* model of more differentiated low risk tumors [5,22], showed ALCAM redistribution to the

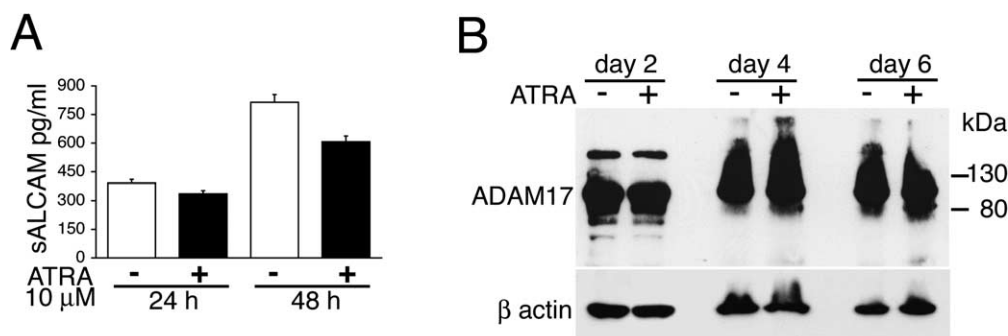


Fig. 3. ATRA treatment of NB cells did not alter ALCAM shedding and ADAM17/TACE expression. (A) Shedding of sALCAM is not increased by ATRA treatment. ELISA detection of sALCAM released by SH-SY-5Y NB cells cultured with 10  $\mu$ M ATRA (black columns) for the indicated times. White columns represent cultures in the presence of the ATRA solvent ethanol. (B) ADAM17/TACE protein expression is not altered by ATRA treatment. SH-SY-5Y cells were treated for the indicated time intervals with either ATRA (10  $\mu$ M) or with solvent only and then analyzed by western blotting with ADAM17/TACE-specific antibodies or with anti- $\beta$ -actin as loading control.

neurites, prompted us to investigate whether different patterns of ALCAM immunostaining could be observed in NB tumors. Immunohistochemistry showed indeed that the ALCAM molecule was expressed in primary tumor tissue and that its expression could be localized at the neuroblast cell membrane and in the neuropil area with different levels of expression in the two compartments, as shown in Fig. 5. It is of note that ALCAM expression in the neuropil, which is formed by non-miellinated neurites and dendrites, was particularly strong in a group of cases.

#### 3.4. ALCAM expression and patients' event-free survival

To evaluate whether different subcellular localizations of ALCAM expression were related to different risks of relapse, an event-free survival (EFS) analysis was performed. We selected 11 tumor specimens from patients with localized resectable disease that experienced relapse, either local ( $N = 6$ ) or metastatic ( $N = 5$ ), despite the absence of known negative prognostic factors [8,9,16,20,26,28,29] and 12 tumors from patients with similar features that never relapsed. ALCAM expression was then used to stratify the 23 patients. When ALCAM expression levels were evaluated by a qualitative score none of the samples were negative and the EFS of the patients was similar regardless of scoring 1, 2 or 3 (data not shown). However, when the semiquantitative grading was independently applied to the membrane and the neuropil area, high ALCAM expression in the cell body membrane significantly associated with worse EFS (Fig. 6A,  $P = 0.0443$ ). In addition, low expression in the neuropil area strongly associated with worse EFS (Fig. 6B,

$P < 0.0001$ ). It is of note that all the patients that showed low ALCAM expression in the neuropil area had also high expression in the cell body membrane. However, no association between site of relapse (local vs. systemic) and ALCAM expression in the different compartments was found.

#### 4. Discussion

In this study we show that NB cell lines display various levels of ALCAM surface expression, which can be dynamically regulated by metalloprotease-mediated shedding, as recently described in other tumors [2,24]. Indeed, in this report we show for the first time that ADAM17/TACE is directly capable to process full-length membrane ALCAM from NB cells to a 65 kDa sALCAM form, which is present also in NB cell supernatants. Intriguingly, the 95 kDa sALCAM form, present in NB cell culture supernatants, was not detectable following *in vitro* digestion. This finding may reflect either the particular *in vitro* conditions required for optimal activity of recombinant ADAM17/TACE (no NaCl and pH 9.0) or the possible involvement of another metalloprotease, still unidentified, which generates the 95 kDa sALCAM form released by living cells.

NB cell lines are usually derived from aggressive, highly proliferating tumors but treatment with retinoic acid can reduce their proliferation *in vitro*, increase their susceptibility to apoptosis and induce a more differentiated phenotype with development of neurites reviewed in [5,22]. It is noteworthy that ALCAM subcellular distribution in NB cells was modified by *in vitro* retinoic acid induced differentiation, as ATRA-

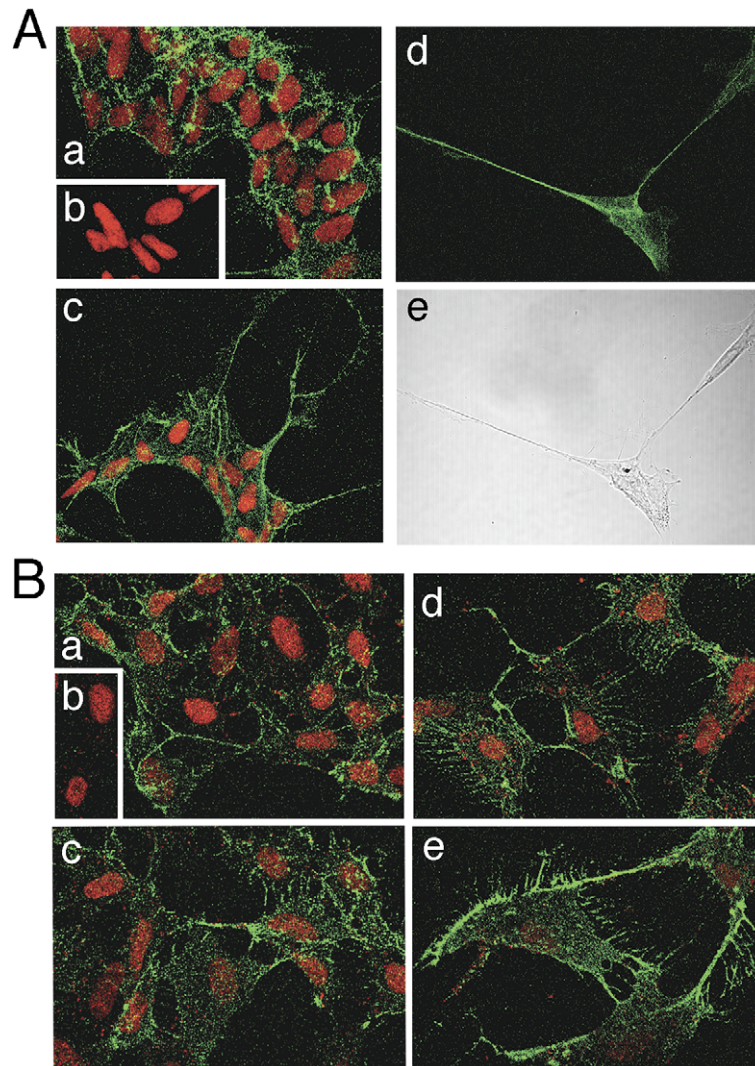


Fig. 4. ALCAM expression and localization in differentiated SH-SY-5Y (A) and SK-N-BE (B) NB cells. Untreated cells (a) and cells treated for 48 h (b, c), 5 (Bd) or 7 days (Ad, Ae and Be) with 10  $\mu$ M ATRA were stained with the anti-ALCAM I/F8 scFv (a, c, d, Be) or with isotype matched control (b) followed by Alexa488-conjugated goat anti-mouse. In Ae, phase contrast image of the same field as in Ad is shown. Nuclei were counterstained with propidium iodide. Immunofluorescence was visualized by confocal microscopy (original magnification 600 $\times$ ).

differentiated NB cells showed strong ALCAM expression on the neurites. However, re-localization of ALCAM to the neurites during ATRA-induced differentiation was not associated with significant changes in ADAM17/TACE levels of expression.

Interestingly, variable levels of expression and different subcellular localizations of ALCAM were found in NB primary tumors. Moreover, in a small but highly uniform cohort of patients with resectable localized NB low ALCAM expression in the neuropil area and high levels in the cell body membrane were associated with relapse, either local or metastatic. Al-

though relapses are rare events in patients with localized NB, taken together these data suggest that the diverse ALCAM subcellular localization may correlate with a different aggressive behaviour in this subset of patients.

It could be speculated that the presence of high ALCAM levels in the neuropil area limit NB cell motility through a homophilic interaction, thus explaining the highest incidence of recurrence in patients with low ALCAM expression in this compartment. In addition, since neuropil is formed by non-myelinated dendrites and neurites, and *in vitro*

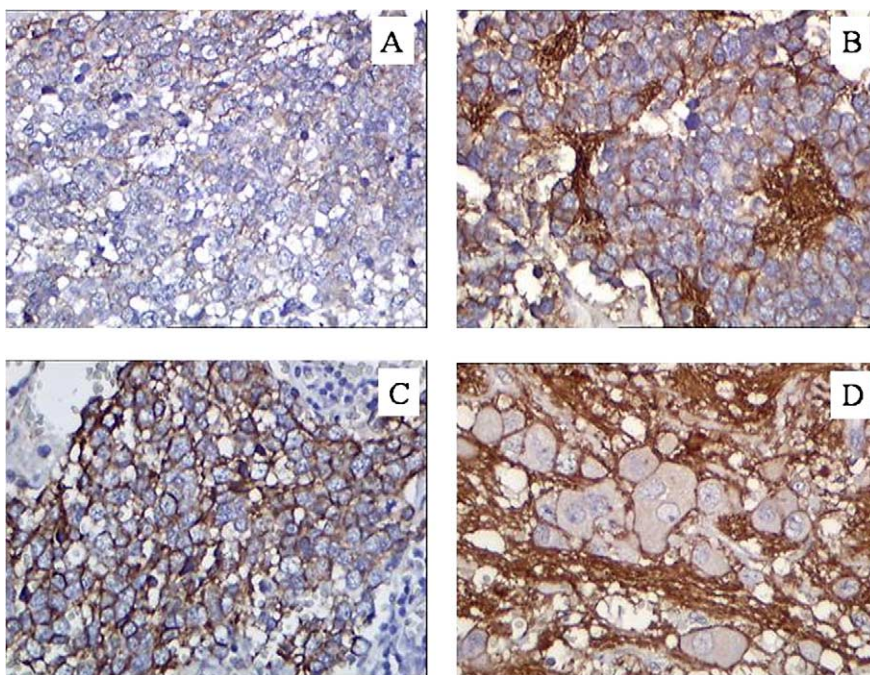


Fig. 5. Expression of ALCAM in NB primary tumors. (A) Low ALCAM expression in membrane and neuropil area. (B) Low ALCAM expression in membrane associated with high expression in neuropil area. (C) High ALCAM expression in membrane associated with low expression in neuropil area. (D) High ALCAM expression in membrane and neuropil area.

ATRA-differentiated NB cell lines showed strong ALCAM expression on neurites, ALCAM staining in the neuropil would identify more differentiated NB tumors, which are expected to correlate with better prognosis.

On the opposite, elevated ALCAM membrane expression on the cell body of neuroblasts appeared to associate with a worse outcome in localized NB. In this context membrane ALCAM may act as pathfinding molecule and support local tissue invasion. Indeed, previous studies indicated a role for ALCAM as pathfinding molecule in the central nervous system, in non-radial cell migration during diencephalic development in chick [13], in retinal axon guidance to the optic disk in goldfish [14,18] and in guiding retinal ganglion cell axons *in vitro* [1]. However, no significant associations between different ALCAM subcellular localization and type of relapse (local or metastatic) were found. Thus, the precise role of ALCAM in mediating either local or metastatic NB invasiveness remains elusive.

Previous studies identified ALCAM cell surface expression as a marker of tumor progression in several tumors, including melanoma, which shares with NB the neuro-ectodermal origin. In melanoma, indeed, ALCAM surface expression correlates with pri-

mary tumor progression and with the invasive vertical growth phase of tumor expansion [33]. Also in colon cancer strong membranous ALCAM staining was associated with shorter survival time [34].

In conclusion, our data demonstrate that high ALCAM expression in the cell body membrane and low ALCAM expression in the neuropil in NB patients with resectable localized disease and favourable biology associate with higher risk of relapse. Thus, the assessment of ALCAM subcellular localization by IHC may represent an easily accessible and useful tool to identify those patients that could benefit from more careful follow-up.

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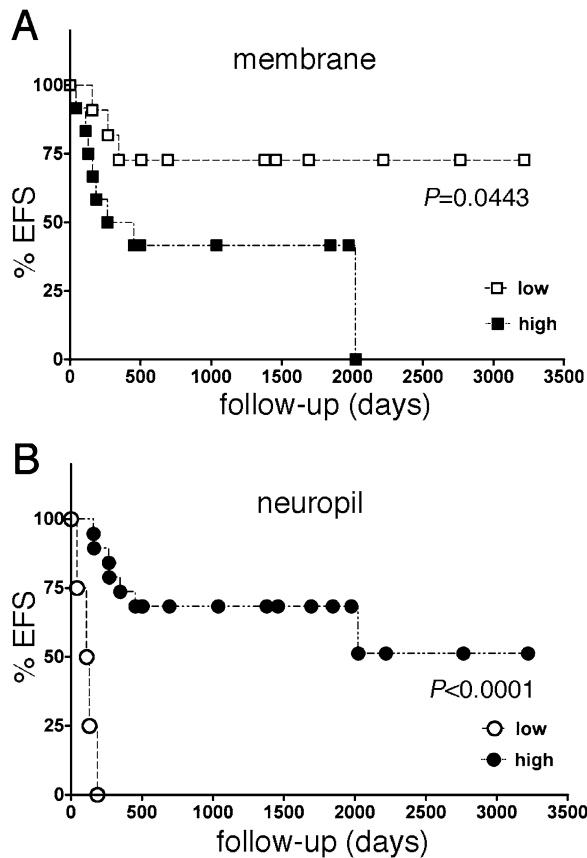


Fig. 6. ALCAM expression and event-free survival of patients with localized NB. Patients were stratified according to the semi quantitative grading of ALCAM expression (low = +/- or +, high = ++ or +++ in membrane (A) or neuropil area (B)). Difference in event free survival (EFS) between patients with high or low ALCAM expression in the membrane was significant ( $P = 0.0443$ ), and difference in EFS between patients with high or low expression in the neuropil area was highly significant ( $P < 0.0001$ ).

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