E-cadherin impairment increases cell survival through Notch-dependent upregulation of Bcl-2

António Carlos Ferreira1,2, Gianpao Suriano1, Nuno Mendes1, Bárbara Gomes1,2, Xiaogang Wen1,2, Fátima Carneiro1,2, Raquel Seruca1,2 and José Carlos Machado1,2,*

1IPATIMUP – Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal and 2Faculty of Medicine, University of Porto, Porto, Portugal

Received August 3, 2011; Revised September 29, 2011; Accepted October 6, 2011

The role of E-cadherin in tumorigenesis has been attributed to its ability to suppress invasion and metastization. However, E-cadherin impairment may have a wider impact on tumour development. We have previously shown that overexpression of mutant human E-cadherin in Drosophila produces a phenotype characteristic of downregulated Notch. Hence, we hypothesized that Notch signalling may be influenced by E-cadherin and may mediate tumour development associated with E-cadherin deficiency.

De novo expression of wild-type E-cadherin in two cellular models led to a significant decrease in the activity of the Notch pathway. In contrast, the ability to inhibit Notch-1 signalling was lost in cells transfected with mutant forms of E-cadherin. Increased Notch-1 activity in E-cadherin-deficient cells correlated with increased expression of Bcl-2, and increased resistance to apoptotic stimuli. After Notch-1 inhibition, E-cadherin-deficient cells were re-sensitized to apoptosis in a similar degree to wild-type E-cadherin cells. We also show that Notch-inhibiting drugs are able to significantly inhibit the growth of E-cadherin-deficient cells xenografted into nude mice. This effect was comparable with the one observed in animals treated with the chemotherapeutic agent taxol, a chemical inducer of cell death.

In conclusion, our results show that aberrant Notch-1 activation, Bcl-2 overexpression and increased cell survival are likely to play a crucial role in neoplastic transformation associated with E-cadherin impairment. These findings highlight the possibility of new targeted therapeutical strategies for the treatment of tumours associated with E-cadherin inactivation.

INTRODUCTION

Even though the incidence of gastric carcinoma (GC) is declining in many Western countries, cancer of the stomach continues to be an important healthcare problem. GC remains the second leading cause of mortality worldwide, within the group of malignant diseases, and accounts for almost 10% of cancer-related annual deaths with 866 000 deaths/year (1). The high mortality results from insufficient efficacy of the available combined treatment, if endoscopic approaches and/or surgery fail. Nowadays, one of the most often applied regimens is the combination of cisplatin and 5-fluorouracil, with docetaxel resulting in about 10 months’ median survival. However, no major breakthrough has been achieved. Addressing novel targets and the application of individualized management should be the way to improve the long-term survival and quality of life for GC patients.

Despite the increased knowledge on the biology of GC development, our understanding of the signalling pathways affected is still quite limited. During the last decade, the role of E-cadherin in tumour progression has been the subject of extensive studies, and it is now generally accepted that alterations in this molecule occur during tumour progression of most carcinomas, namely gastric carcinoma (2–7). More than 70% of all GCs lose E-cadherin along tumour progression and this is correlated with poor prognosis and increased metastization. CDH1 germline inactivation causes a hereditary form of GC, coined hereditary diffuse gastric cancer (HDGC) (8–10). In this syndrome, diffuse GC displays abnormal or absent E-cadherin protein expression caused by germline
point mutations and large deletions of the CDH1 gene (5). Recently, we also showed CDH1 germline allelic expression imbalance in 70% of CDH1-negative probands. Importantly, these observations implicate E-cadherin as the molecular culprit in the vast majority of HDGC families (mutation-positive and mutation-negative) (11).

An apparently attractive approach for disease treatment would be the reconstitution of E-cadherin expression. Nevertheless, this would only be possible in cases where E-cadherin can be re-expressed at cell membrane by the use of demethylating agents, in case of promoter hypermethylation, or the use of chemical chaperons as previously reported for specific E-cadherin mutations (12). However, this pharmacological approach poses significant limitations to apply into clinics, since it affects other gene promoters besides E-cadherin, thus being an unspecific treatment. The identification of E-cadherin molecular targets is therefore mandatory. Despite that, the signalling pathways that are aberrantly activated in consequence of E-cadherin loss, observed in the progression of epithelial cancers, remain elusive in a significant percentage of cases.

Using an in vivo model system, we have expressed human E-cadherin mutants in Drosophila and found that one of them produced a phenotype characteristic of downregulated Notch (13), calling attention for the possibility that E-cadherin may interfere with Notch signalling. Hence, in this work we hypothesized that Notch signalling may be influenced by E-cadherin and mediate tumour development associated with E-cadherin deficiency.

**RESULTS**

**E-cadherin impairment associates with increased Notch-1 signalling**

In order to verify whether E-cadherin loss-of-function was associated with the activation of Notch pathway, we determined the level of expression of Notch-1 and Notch intracellular domain (NICD) in E-cadherin wild-type versus its mutant forms. We transduced a human epithelial non-expressing E-cadherin cell line (MDA-MB-435) with the two HDGC-related germline mutations T340A and V832M, or with the wild-type E-cadherin for comparison. The T340A alteration (A → G in nucleotide 1018 of the CDH1 gene) affects the extracellular domain of the protein, whereas the V832M mutation (G → A in nucleotide 2494) localizes to the cytoplasmatic tail. Both mutations lead to loss-of-function of the protein, impairing cell–cell adhesion and decreasing the ability to suppress cell invasion (10,14).

We found an inverse association between the expression of wild-type E-cadherin and Notch pathway activation, measured by the accumulation of its active form, NICD (Fig. 1A). The wild-type E-cadherin cell line showed almost absent NICD levels in contrast to Mock cells. The wild-type E-cadherin cell line showed almost absent NICD levels in contrast to Mock cells. We also observed differences in Notch-1 mRNA (Fig. 1B) and full-length Notch-1 protein expression between Mock and wild-type E-cadherin cells (Fig. 1A). Interestingly, cells expressing the mutated forms of E-cadherin behaved in a similar way to Mock cells. In both E-cadherin mutants, the level of expression of Notch-1 and NICD was higher when compared with E-cadherin wild-type cells at mRNA and protein levels (Fig. 1A and B).

Our in vitro results were validated in vivo using a small series of primary GCs. The expression of Notch-1 and E-cadherin was compared in four sporadic early-stage signet-ring-cell carcinomas by immunohistochemical methods. Interestingly, in the single case with complete loss of E-cadherin expression in tumour cells, strong Notch-1 expression was observed (Fig. 2).

**Increased Notch-1 activity leads to Bcl-2 overexpression**

We investigated in vitro the downstream effects of elevated NICD levels on the Notch canonical target genes of the Hes-and Hey-family (15,16). No differences were observed in Hes-1, Hey-1 and Hey-L amounts between Mock, mutant and wild-type E-cadherin cell lines (Fig. 3). Expression of Hes-5 and Hey-2 was not detected in any cell line (data not shown). Altogether, our results suggest that, in this specific
model, the classical Notch-1 targets were excluded and not related to the increased Notch pathway activity.

Additionally, we investigated other candidate downstream targets of increased Notch-1 activation, namely proteins that have been associated with Notch in the development of solid tumours (17–21). No changes in expression were observed for NF-κB, PI3K, AKT, ERKs, cyclin-D1, p27, p21, XIAP and c-Myc (data not shown). Interestingly, we found an association between E-cadherin loss-of-function (Mock and E-cadherin mutant cells), Notch pathway activation and increased expression of BCL-2. The opposite was found in wild-type E-cadherin cells (Fig. 3). These novel results are in accordance with previous data from our group (22), showing that E-cadherin loss-of-function was correlated with increased levels of BCL2 expression.

It has been described that caspase activation can induce degradation of Bcl-2 (23). To confirm that the lower amounts of Bcl-2 observed in wild-type E-cadherin cells were due to lower Notch activation and not to increased caspase activation, we treated cells with Z-VAD, a pan-caspase inhibitor (24). Inhibition of caspase activity was confirmed by the decrease in the levels of cleaved PARP, one of the first targets of caspases. However, it did not influence Bcl-2 amounts (Fig. 5), indicating that Bcl-2 accumulation was independent of the caspase activation status.

Altogether, our results demonstrate that loss of E-cadherin function, by mutational events or absence of expression (Mock cells), impairs its ability to suppress the activation of Notch signalling, leading to an accumulation of the anti-apoptotic protein Bcl-2.

**Notch inhibition overcomes apoptosis resistance in E-cadherin-mutant cells**

In order to determine the cellular consequences of increased Bcl-2 expression in non-functional E-cadherin cells, we studied cell death rates and apoptosis by TUNEL assays. All cell lines (E-cadherin wild-type or mutant), in the absence of an apoptosis stimulus, showed similar levels of apoptosis (Fig. 6, yellow bars). Under the same conditions, treatment with Notch-1 siRNA led to a comparable increase in the apoptosis level in all four cell lines (Fig. 6, pink bars). In contrast, when cells were cultured in the presence of an apoptosis stimulus (serum starvation), E-cadherin-deficient cells were more resistant to apoptosis than E-cadherin wild-type cells: wild-type E-cadherin cells versus E-cadherin T340A, \( P = 0.0004 \); versus E-cadherin V832M, \( P = 0.0002 \); versus Mock...
cells, \( P = 0.0002 \) (Fig. 6, blue bars). However, when cells were treated with Notch-1 siRNA, the resistance to apoptosis was abrogated rendering E-cadherin-deficient cells sensitive to apoptosis in a level comparable with E-cadherin wild-type cells (Fig. 6, red bars).

Similar results were obtained when Notch-1 was inactivated using the pharmacological drug DAPT, a chemical compound that inhibits the activity of \( \gamma \)-secretase/presenilin complex (25). This complex is responsible for the final cleavage (S3) of the Notch receptor that enables the release of NICD from the membrane to the nucleus (26). Blockage of \( \gamma \)-secretase results in the absence of Notch-1 activation, reduction of NICD levels and accumulation of NEXT (Notch extracellular truncated), a protein form that only has about 30 more amino acids than NICD but is still localized at the membrane and lacks signalling properties.

Treatment with DAPT was successful in inhibiting Notch-1 activation in non-functional E-cadherin cells. This was shown by NICD loss and accumulation of NEXT (Fig. 7A). Moreover, treatment with DAPT also led to Bcl-2 downregulation in E-cadherin-deficient cell lines (Fig. 7A). Therefore, we determined whether inhibition of Notch-1 by DAPT was also able to overcome the apoptotic resistance observed in E-cadherin-deficient cells. First, we verified that, in the absence of apoptosis stimulation (presence of serum), DAPT treatment increased apoptosis in a similar rate in all cell lines independently of the E-cadherin status (Fig. 7B, pink bars).

In contrast, in serum-free conditions, DAPT treatment demonstrated that the \( \gamma \)-secretase inhibitor was efficient in increasing cell death in E-cadherin-deficient apoptosis-resistant cells to the same rates observed in wild-type E-cadherin cells, thus re-sensitizing cells for apoptosis (Fig. 7B, red bars). In fact, under serum starvation conditions, E-cadherin-deficient cells showed equivalent level of resistance to apoptosis when compared with wild-type E-cadherin cells; wild-type E-cadherin cells versus E-cadherin T340A, \( P = 0.0008 \); versus E-cadherin V832M, \( P = 0.0007 \); versus Mock cells, \( P = 0.0008 \); (Fig. 7B, blue bars).

These results demonstrate that E-cadherin-deficient cells are resistant to apoptotic stimuli, and that this resistance is dependent on Notch signalling.

**Notch inhibition sensitizes E-cadherin-deficient cells to cell death in vivo**

To evaluate whether the effects associated with Notch inhibition in vitro in E-cadherin-deficient cells were confirmed in vivo, we generated tumour xenografts by injecting CHO cells, transfected with wild-type E-cadherin or the empty vector, in N:NIH (S) nu/nu mice, and treated the animals with DAPT. For this specific experiment, we used CHO cells due to their ability to generate tumours in vivo (27).

Prior to this experiment, we demonstrated that, in CHO cells, Notch-1 activity is also inhibited by wild-type E-cadherin, and that DAPT treatment is efficient in inhibiting Notch activation in cells with non-functional E-cadherin (Supplementary Material, Fig. S2). Mock and wild-type E-cadherin cells were used because these are the extremes of the spectrum.
(presence or total absence of E-cadherin). For each cell line, inoculation of $1 \times 10^6$ cells was performed subcutaneously in 8–10-week-old male N:NIH (S) nu/nu mice and starting at day 3 post-inoculation, the animals were treated daily with subcutaneous injections of DMSO or DAPT (10 mg/kg), or once every 3 days with paclitaxel/taxol (10 mg/kg). During the experiment, non-inoculated mice and mice inoculated with wild-type E-cadherin cells did not reveal any tumour growth. In addition, no weight loss or any other macroscopic effects were observed in non-inoculated animals treated with DAPT or taxol.

In animals inoculated with E-cadherin-negative cells (CHO Mock cells), tumours were noticeable from post-inoculation day 6 and grew until a mean value of 1356 mm$^3$ at day 19 (Fig. 8, yellow). In animals treated with DAPT, there was a statistically significant reduction in tumour growth (Fig. 8, blue): day 17, DMSO versus DAPT, $P = 0.04$; day 19, DMSO versus DAPT, $P = 0.04$. The effect achieved by the $\gamma$-secretase inhibitor was similar to the one observed in animals treated with taxol (Fig. 8, red). The taxol group was used as a standard for tumour growth inhibition, as taxol is a well-known chemotherapeutic agent that also blocks the development of tumour xenografts (28). Therefore, our results indicate that Notch inhibition is also effective in vivo in reducing the tumorigenic growth of E-cadherin-deficient cells.

**DISCUSSION**

More than 70% of all GCs lose E-cadherin along with tumour progression and this is correlated with poor prognosis and increased metastatization. The CDH1 gene can be genetically inactivated (deregulated) by a number of mechanisms. Somatic mutations of CDH1 were reported in sporadic forms of diffuse-type GC clustering in the extracellular domain of the protein (2). Intragenic deletions can also lead to gene silencing (29). In 2001, Machado et al. (3) showed that CDH1 promoter methylation occurs in 50% of sporadic diffuse-type GC and Carvalho et al. (4) showed that promoter methylation can also occur in intestinal type GC, leading to E-cadherin loss-of-function.

E-cadherin germline mutations are causal events in cases of HDGC (8,30). In vitro and in vivo functional assays have...
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vations are modulated intracellular signalling and influences several key pathways. The lack of this knowledge precludes important therapeutic advances to survey patients with carcinomas harbouring alterations of E-cadherin. Aberrantly activated signalling molecules interacting with E-cadherin represent the ideal targets for the development of therapeutic agents.

In a Drosophila model, expression of a mutant human E-cadherin on the wing disc resulted in the thickening of the distal vein and notching of the wing margin, suggesting that E-cadherin may modulate Notch signalling. The evolutionarily conserved Notch pathway is essential during development, plays an important role in cell fate determination and has multiple post-natal homeostatic functions in the maintenance of normal tissues. Notch signals can affect numerous cellular functions related to tumorigenesis such as proliferation, apoptosis, differentiation, angiogenesis and seem to be re-acquired in different forms of cancer (17,18).

Our findings show the existence of a negative association between E-cadherin function and Notch-1 activation in human cells, similar to what was observed in the Drosophila model. This was demonstrated by the significant decrease in the activation of Notch pathway upon transduction of the wild-type E-cadherin into MDA-MB-435 cell line (cell line lacking E-cadherin expression). The same was not found when MDA-MB-435 cell line was transduced with non-functional mutant forms of E-cadherin. Several recent publications have alluded to the existence of such molecular interaction (31–34). Stylianou et al. (31) showed elevated Notch signalling in correlation with E-cadherin absence in a panel of human breast cancer cell lines. Furthermore, the authors showed that constitutively activated Notch reduced E-cadherin expression, whereas introduction of an inhibitor of Notch, Numb, was sufficient to decrease NICD and significantly increase E-cadherin levels (31). Other authors showed that Jagged-1-mediated activation of Notch-1 can lead to the upregulation of Slug, a transcriptional repressor of E-cadherin (32). In line with our own findings, inhibition of ligand-induced Notch signalling in xenografted Slug-positive/E-cadherin-negative breast tumours promoted apoptosis, inhibited tumour growth and metastasis and associated with downregulated Slug expression and re-expression of E-cadherin (32).

In our model, increased Notch-1 signalling was associated with increased expression of Bcl-2, an anti-apoptotic protein that can prevent apoptosis by directly limiting the action of the pro-apoptotic members of the Bcl-2 family (35). This association was confirmed upon downregulation of Notch-1 by siRNA treatment. In this case, decreased levels of Notch-1 were accompanied by a similar decrease in Bcl-2 levels. In an experiment set-up to test the association between E-cadherin impairment, Notch-1 signalling, Bcl-2 expression and apoptosis resistance, we showed that wild-type E-cadherin cells were significantly more sensitive to apoptosis induction than cells lacking a functional E-cadherin (mutant and cells negative for E-cadherin). Moreover, apoptosis resistance was ‘blocked’ by inhibiting Notch-1 signalling using siRNA strategies or DAPT treatment.

Our results suggest that in addition to its role in loss of cell–cell adhesion, the oncogenic potential of E-cadherin loss-of-function can be also attributed to an increase in cell survival, caused by the aberrant activation of Notch-1 and upregulation of Bcl-2. Indeed, inhibition of apoptosis has been described in gastric tumours, and increased expression of Bcl-2 has also been reported in diffuse GC (36,37). Furthermore, in a series of GCs, Bcl-2 expression was correlated with the presence of E-cadherin mutations (38). In contrast to the well-established contribution of E-cadherin to gastric tumour development, the Notch pathway has not been well characterized in gastric tissues and reports on the subject are scarce. However, Notch-1 overexpression has been reported in GC patients: Li et al. (39) showed that Notch-1 positivity correlated with tumour size, depth of invasion, differentiation grade and poor prognosis. It is worth noting that in primary tumours we found that tumour cells without E-cadherin expression have also Notch-1 de novo expression.

The most interesting finding of our results is the putative therapeutic value of Notch-1 in gastric tumours. In fact, Notch signalling inhibition (through several different strategies) has been correlated to reversion of the transformed phenotype in cancer cell lines, growth arrest of primary tumours and distant metastases and to in vivo tumour regression (31,32,40). Our findings are in accordance with these observations, and suggest that E-cadherin impairment (either by loss of expression or mutation) leads to increased Notch-1 signalling and increased cell survival, but upon apoptosis stimulation, Notch inactivation can overcome the apoptotic resistance observed in E-cadherin-deficient cells. Our in vitro results were further supported by our in vivo results. In this work, we also showed that a drug that impairs Notch-1 pathway activation (DAPT) was able to significantly inhibit the growth of E-cadherin-deficient cells xenografted into human xenografts in male N:NIH (S) nu/nu mice, treated with vehicle (filled triangle), DAPT (cross, 10 mg/kg body weight) or taxol (filled circle, 10 mg/kg). *P = 0.04, comparison between DMSO and DAPT at post-inoculation day 17; **P = 0.04, comparison between DMSO and DAPT at post-inoculation day 19.

Figure 8. Growth of CHO Mock xenografts in male N:NIH (S) nu/nu mice, treated with vehicle (filled triangle), DAPT (cross, 10 mg/kg body weight) or taxol (filled circle, 10 mg/kg). *P = 0.04, comparison between DMSO and DAPT at post-inoculation day 17; **P = 0.04, comparison between DMSO and DAPT at post-inoculation day 19.
nude mice. All these findings highlight the therapeutic value of drugs such as DAPT for gastric cancer treatment and the possibility of new targeted drug strategies for the treatment of gastric carcinogenesis associated with the inactivation of the E-cadherin gene, combining Notch inhibitors with apoptotic stimulators, like the commonly used apoptotic agent taxol. The identified therapeutic targets will allow designing new initial clinical studies by the combination of conventional and new interventions. E-cadherin- and NOTCH pathway-tailored treatments are expected to play the key role in these activities.

MATERIALS AND METHODS

Construction of plasmids encoding human wild-type and mutant E-cadherin forms

Human wild-type and two HDGC-associated germline missense-mutated (T340A and V832M) E-cadherin cDNA fragments were generated as previously described (10,14). These fragments were cloned into the pLent6/V5 Directional TOPO vector (Invitrogen) for retroviral infection, following the manufacturer’s instructions.

Establishment of cell lines stably expressing the different E-cadherin constructs

The E-cadherin-negative MDA-MB-435 cells were chosen as cell model system. Retroviral infection was performed using the Virapower infection kit (Invitrogen), according to the manufacturer’s instructions. 293FT cells were chosen for viral packaging and lipofectamine transfection (Invitrogen) performed with 95% confluent cells in OPTI-Mem medium (GIBCO-BRL). Virus-containing supernatants were collected at 48 and 72 h, respectively, upon transfection, filtered and applied, in the presence of 100 μg of polybrene, on MDA-MB-435 target cells grown at 60% confluence. Blasticidin selection (5 μg/ml; Invitrogen) was carried out for the following 3 weeks. To verify the expression of E-cadherin in the selected cells, western blotting and immunocytochemistry were performed.

MDA-MB-435 cells transduced with wild-type or mutated forms of E-cadherin (T340A and V832M) were maintained in Dulbecco’s modified Eagle’s medium (GIBCO, Invitrogen), supplemented with 10% fetal bovine serum (FBS; GIBCO, Invitrogen), 100 μg/ml penicillin/streptomycin (Invitrogen) and under blasticidin selection in a 10% CO₂ humidified atmosphere at 37°C. As negative control, MDA-MB-435 cells were transduced with the empty vector (Mock).

We also analysed E-cadherin-negative CHO-K1 (Chinese hamster ovary) cells transfected with wild-type or mutated forms of E-cadherin (T340A and V832M) were established as described elsewhere (10,14). Cells were grown in α-MEM (+) medium (GIBCO, Invitrogen), supplemented with 10% FBS, 100 μg/ml penicillin/streptomycin and under Geneticin G418 sulphate selection (500 μg/ml; Invitrogen) in a 5% CO₂ humidified atmosphere at 37°C.

Antibodies

The following primary antibodies were used: anti-E-cadherin (BD Transduction Laboratories, 610182); anti-Notch-1 (Santa Cruz Biotechnology, SC-6014); anti-Jagged-1 (Santa Cruz Biotechnology, SC-6011); anti-Jagged-2 (Cell Signaling, 2205); anti-Delta-1 (Santa Cruz Biotechnology, SC-9102); anti-Delta-3 (Cell Signaling, 2483); anti-Delta-4 (Cell Signaling, 2589); anti-β-catenin (Sigma, C2206); anti-Hes-1 (kind gift from Dr Tetsuo Sudo, Toray Industries, Inc.); anti-Hey-1 (Santa Cruz Biotechnology, SC-16424); anti-Hey-L (Santa Cruz Biotechnology, SC-16448); anti-Hey-2 (Chemicon, AB5716); anti-Hes-5 (Chemicon, AB5708); anti-Bcl-2 (Dako, M0887); anti-p21 (BD Transduction Laboratories, 554228); anti-p27 (Santa Cruz Biotechnology, SC-528); anti-cyclin-D1 (Santa Cruz Biotechnology, SC-20044); anti-NF-kappaB p65 (Santa Cruz Biotechnology, SC-372); anti-phospho-NF-kappaB p65 (Cell Signaling, 3031); anti-AKT (Cell Signaling, 2966); anti-phospho-AKT (Cell Signaling, 4051); anti-ERK1/2 (Cell Signaling, 9102); anti-phospho-ERK1/2 (Cell Signaling, 9106); anti-XIAP (Cell Signaling, 2042); anti-Pi3K (BD Transduction Laboratories, 610046); anti-c-Myc (Santa Cruz Biotechnology, SC-42); anti-PARP (Santa Cruz Biotechnology, SC-7150). Anti-α-tubulin (Sigma, T6199) was used as loading control.

Preparation of cell lysates, immunoblotting and immunoprecipitation

Cells were lysed using cold catenin lysis buffer [1% Triton X-100, 1% Nonidet P-40 with 1:7 proteases inhibitor cocktail (Roche) and 1:100 phosphatases inhibitor cocktail (Sigma)]. Protein concentration was determined using the Lowry Assay (BioRad DC reagents, BioRad).

Prior to SDS–PAGE, proteins were dissolved in the sample buffer (90% of Laemmli, 5% of 2-β-mercaptoethanol and 5% of Bromophenol Blue) and heated for 5 min at 95°C. After electrophoresis, proteins were transferred onto Hydro nitrocellulose membranes (GE Healthcare). Membranes were blocked with 4% bovine serum albumin (Sigma) in PBS + 0.5% Tween-20 (for the detection of phosphorylated proteins) or with 5% non-fat milk in PBS + 0.5% Tween-20 (for overall protein detection) and incubated overnight with the primary antibody specific for the protein of interest. Anti-goat (Santa Cruz Biotechnology, F2206), anti-mouse (GE Healthcare, NA931V) or anti-rabbit (GE Healthcare, NA934V) horseradish peroxidase-conjugated secondary antibodies were used, followed by ECL western blotting detection (GE Healthcare).

For immunoprecipitation assays, 600 μg of proteins were incubated with primary antibody ON at 4°C. Immuno-complexes were then incubated for 60 min with protein G-Sepharose beads (GE Healthcare), washed, eluted in sample buffer, heated for 5 min at 95°C and submitted to SDS–PAGE and immunoblotting.

siRNA transfection and DAPT treatment

siRNAs targeting Notch-1 mRNA were obtained from Qiagen (Hs_Notch1_2 cat SI00119021; Hs_Notch1_3 cat SI00119028) and prepared according to the manufacturer’s
instructions. As negative control, non-silencing siRNA (All Stars Negative Control cat 1027281, Qiagen) was used. Prior to transfection, 1.5 x 10^5 cells were grown in six-plate wells for 24 h. Afterwards, cells were transiently transfected with 125 nm siRNA, using the Lipofectamine 2000 transfection reagent (Invitrogen) and incubated with 10% FBS and antibiotic-free medium. After 12 h, medium was removed and replaced with fresh antibiotic-free medium that could either contain 10% FBS or be serum-free according to the desired experiment.

DAPT (N-[3-(4-difluorobenzoyl)-(1R,2S)-phenylacetic acid])-L-alanyl-S-phenylglycine t-butyler ester was purchased from Sigma (D5942) and dissolved in DMSO to a working solution of 10 mM. For DAPT treatment, cells were grown in six-well plates and 1 μl of the working solution was added to each millilitre of medium. For control samples, the same volume of DMSO (1 μl) was added to each millilitre of medium.

**RNA extraction and RT-PCR**

Total RNA extraction was performed with Tripure isolation reagent (Roche), according to the manufacturer’s instructions. The isolated RNAs were treated with ribonuclease-free deoxyribonuclease I (1 U/μl). Total RNA extraction was performed with Tripure isolation reagent (Invitrogen) and incubated with 10% FBS and antibiotic-free medium. After 12 h, medium was removed and replaced with fresh antibiotic-free medium that could either contain 10% FBS or be serum-free according to the desired experiment.

RT-PCR assays were done using Quantitect SYBR Green PCR kit (Qiagen), with SYBR Green I as the fluorescent dye enabling real-time detection of PCR products according to the manufacturer’s protocol. Notch-1-specific primers (Quantitect Primer Assay Hs_Notch1_2_SG cat QT01005109, Qiagen) were purchased from Qiagen. Reactions were performed in an ABI Prism 7000 SDS v1.1, using the following conditions: 95°C for 15 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 1 min. For quantification, relative gene expression of the target gene was determined after normalization to the internal standard 18S gene.

**Caspase inhibition/Z-VAD treatment**

The pan-caspase inhibitor Z-VAD (Z-Val-Ala-Asp-fluoromethylketone) was obtained from Bachem AG (N-15110). The drug was dissolved in methanol to a working solution of 10 μM. Treatment was performed by adding 1 μl of the working solution for each millilitre of medium to cells grown in six-well plates, for 48 h. For control samples, the same amount of methanol was added.

**TUNEL assay**

In situ cell death detection kit (Roche) was used to detect apoptotic cell death by enzymatic labelling of DNA strand breaks. After treatment (siRNA or DAPT), cells were fixed in 4% paraformaldehyde for 20 min at room temperature and cyto spun into slides. Slides were washed in PBS, and cells were then suspended in permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 3 min on ice. Cells were washed again and treated with 30 μl of TUNEL-reaction mixture for 1 h in a humidified dark chamber at 37°C.

Following PBS washing, slides were mounted with Vectashield mounting medium with 4′-6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Green fluorescence of apoptotic nuclei was detected by fluorescence microscopy. For each slide, a minimum of 2000 nuclei were counted.

**Slow aggregation assay**

Each well of 96-well plate was coated with 50 μl of a semi-solid agar-solution (100 mg of Bacto-agar (Difco Laboratories) in 15 ml of sterile PBS, dissolved at 40–50°C). On each well, 2 x 10^4 trypsinized cells were seeded and the plate incubated at 37°C in a humidified atmosphere with 5% of CO2 for 48 h. Aggregation was evaluated under an inverted microscope (4x objective) at 24 and 48 h.

**Tumour xenograft model**

CHO Mock or E-cadherin wild-type cells (1 x 10^6) in 100 μl of PBS were inoculated subcutaneously into 8–10-week-old male N:NIH (S) nu/nu mice. At post-inoculation day 3, groups of five animals each were treated subcutaneously everyday with 100 μl of 50% DMSO/H2O (vehicle group), 10 mg/kg of body weight of DAPT, or once every 3 days with 10 mg/kg of body weight of taxol/paclitaxel. Tumour volume (in mm^3) was calculated using the formula (length x width^2)/2. As controls, groups of non-inoculated animals were treated under the same conditions with DMSO, DAPT or taxol. At the end of the study, animals were humane euthanized.

**Immunohistochemistry**

Surgical specimens from four early diffuse signet-ring carcinomas were resected and diagnosed at Hospital Sáo João, Porto, Portugal. Tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 3 μm were obtained from each block and used for haematoxylin and eosin staining and immunohistochemistry.

Antigen retrieval was performed in Trilogy buffer (Cell Marque) for 20 min at 99°C. Non-specific endogenous peroxidase activity was eliminated by DAKO Peroxidase Block (Dako ENVision+ System, Peroxidase, Dako) for 10 min at room temperature. Slides were washed in water and PBS-Tween, and incubated with rabbit anti-Notch-1 (Santa Cruz, SC-6014, 1:100 dilution) or mouse E-cadherin (Zymed Laboratories, 13-1700, 1:50 dilution) primary antibody for 1 h at room temperature. After washing, slides were incubated with horseradish peroxidase-labelled polymer that is conjugated to rabbit and mouse secondary antibodies (Dako ENVision+ System, Peroxidase, Dako) for 30 min at room temperature. Slides were then washed and staining was completed by a 5–10 min incubation with 3,3′-diaminobenzidine (DAB) and substrate-chromogen (Dako ENVision+ System, Peroxidase, Dako). Finally, slides were counterstained with haematoxylin, dehydrated and mounted.


