N-cadherin increases after androgen deprivation and is associated with metastasis in prostate cancer

Karin Jennbacken, Tajana Tešan, Wanzhong Wang,
Heléne Gustavsson, Jan-Erik Damber and Karin Welén

Lundberg Laboratory for Cancer Research, Department of Urology, Institute of Clinical Sciences, Sahlgrenska Academy at University of Gothenburg, Sahlgrenska University Hospital, Gula Stråket 8, SE-413 45 Göteborg, Sweden

(Correspondence should be addressed to K Welén; Email: karin.welen@urology.gu.se)

Abstract

Androgen-deprivation therapy (ADT) is the standard treatment for metastatic prostate cancer. One factor that has been implicated in the metastatic process is the cell adhesion molecule N-cadherin. In this study, we investigated if the expression of N-cadherin was influenced by androgen deprivation and was associated with metastasis in prostate cancer. The effect of androgen deprivation on N-cadherin expression was initially studied in androgen-dependent (AD) LNCaP and androgen-independent (AI) LNCaP-19 and PC-3 prostate cancer cell lines. Expression of N-cadherin increased in the absence of androgens in AI LNCaP-19 primary tumors and metastases and also *in vitro*, but not in AI PC-3 tumors, indicating a possible involvement of the androgen receptor in the regulation of N-cadherin. N-cadherin was absent in AD LNCaP tumors. No clear associations between N-cadherin and factors related with epithelial–mesenchymal transition or neuroendocrine differentiation could be established. In addition, N-cadherin was evaluated by immunohistochemistry in human prostate tumors. Expression of N-cadherin was more frequently found in tumors from patients treated with ADT than in tumors from patients with no prior hormonal treatment. N-cadherin expression was also associated with metastasis and Gleason score. Furthermore, increased N-cadherin was detected in prostate cancer biopsies already 3 months after initiation of ADT when tumors were in a regressed state. In summary the results indicate that androgen deprivation induces N-cadherin in prostate tumors. Moreover, N-cadherin was increased in castration-resistant tumors in patients with established metastases. This might indicate that castration induces molecular alterations in the tumor cells, resulting in a more invasive and metastatic phenotype.

Endocrine-Related Cancer (2010) 17 469–479

Introduction

Most cancer deaths are due to metastatic disease. The metastatic process involves several steps, including detachment from the primary tumor, migration, intravasation into vessels, and establishment at a new site (Chambers *et al.* 2002). N-cadherin is one factor that has been implicated in the metastatic process. In normal tissue, N-cadherin is highly expressed in the nervous system, but it is also found in the vascular endothelium and myocardium (Hatta *et al.* 1987, Salomon *et al.* 1992). It is located at adherens junctions, and with its extracellular domain, it mediates a dynamic contact between cells and between cells and

matrix (Hazan *et al.* 1997). In addition, the cytoplasmic domain is involved in multiple intracellular signaling pathways (Derycke & Bracke 2004). In several human cancers, expression of N-cadherin is found to be elevated (Derycke & Bracke 2004). Increased expression of N-cadherin is, together with the loss of the tumor suppressor E-cadherin, one feature of epithelial–mesenchymal transition (EMT) that takes place during cancer progression, and this ‘cadherin switching’ plays an essential role in the motility of cancer cells (Maeda *et al.* 2005). The EMT process is also clearly of importance for metastasis of prostate cancer (Xie *et al.* 2010). In contrast to N-cadherin, E-cadherin mediates homotypic interactions between

cells, which maintain the integrity of epithelial tissues (Giehl & Menke 2008). Functional studies show that N-cadherin makes tumor cells more motile, and promotes invasiveness and metastasis in experimental models of cancer (Hazan *et al.* 1997, 2000, Li *et al.* 2001). The most possible mechanism by which N-cadherin renders tumor cells more motile is through the homophilic adhesion to other cells. First, N-cadherin mediates a dynamic cell adhesion resulting in weaker interactions between adjacent cells than E-cadherin (Chu *et al.* 2004). Thus, it allows the dissociation of single cells from the primary tumor. Secondly, homophilic interactions between tumor cells and N-cadherin-expressing tissues, such as the stroma and vasculature, facilitate the transit through the tissue and survival of tumor cells in distant organs (Sandig *et al.* 1997). Moreover, the invasive capacity of N-cadherin is in part due to an interaction with the fibroblast growth factor receptor 1 (Suyama *et al.* 2002). In addition to the prometastatic properties of N-cadherin, aberrant expression in cancer has also been shown to inhibit apoptosis (Li *et al.* 2001, Tran *et al.* 2002) and promote angiogenesis (Derycke *et al.* 2006a,b). An N-cadherin antagonist, ADH-1 (Exherin), has been evaluated in experimental models of cancer, and is at present in clinical trials for treatment of solid tumors (Augustine *et al.* 2008, Shintani *et al.* 2008, Beasley *et al.* 2009, Perotti *et al.* 2009).

In a previous study, we reported that a switch from E-cadherin to N-cadherin was associated with the transition of androgen-dependent (AD) LNCaP into androgen-independent (AI) LNCaP-19 prostate cancer cells (Jennbacken *et al.* 2006). This switch has also been reported to occur in other AI cell lines (Tran *et al.* 1999, Bussemakers *et al.* 2000). In human prostate cancer, N-cadherin expression has been found in poorly differentiated areas (Bussemakers *et al.* 2000, Tomita *et al.* 2000), and its expression correlated with Gleason score (Jaggi *et al.* 2006). In a recent study, it was shown that N-cadherin expression in human prostate tumors was associated with pelvic lymph node infiltration and shorter time to skeletal metastasis (Gravdal *et al.* 2007).

The established treatment of locally advanced and metastatic prostate cancer is androgen-deprivation therapy (ADT). ADT is initially successful, resulting in reduced tumor burden that could last for several months or even years. However, eventually an AI tumor (i.e. castration-resistant tumor) with an aggressive and metastatic phenotype relapses. Today, treatment in the castration-resistant stage is never curative, only palliative, and includes continued hormonal

therapy, radiation, or cytotoxic treatment (Damber & Aus 2008). Transition into androgen independency is not clearly understood, but several mechanisms involving the androgen receptor (AR) have been suggested to be of importance (Marcelli *et al.* 2000, Gregory *et al.* 2001, Linja *et al.* 2001). To improve treatment in the castration-resistant stage, there is a need to identify regulators of aggressive and metastatic prostate cancer and clarify which molecular alterations are induced by ADT.

In contrast to E-cadherin, the expression of N-cadherin in prostate cancer is not extensively studied, and there is no study addressing the association of N-cadherin with tumor progression after ADT. The objective of the present study was to investigate how the expression of N-cadherin was influenced by androgen deprivation, both in prostate cancer cell lines and in human prostate tumors.

Materials and methods

Cell lines and culture conditions

LNCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and its subline LNCaP-19 was previously established in our laboratory (Gustavsson *et al.* 2005). PC-3 cells were obtained from the European Collection of Cell Cultures (ECCC, Wiltshire, UK). Cells were maintained as described previously (Tesan *et al.* 2008).

For RNA analyses, cells were cultured for 10 days in 10% steroid depleted dextran charcoal treated FBS (DCC-FBS), without or with the addition of the synthetic androgen R1881 (NEN Life Science Products, Inc., Boston, MA, USA) in different concentrations (0.1 and 1 nM). In addition, a proportion of the LNCaP-19 cells were first cultured in the absence of androgens for 10 days followed by androgen stimulation (0.1 or 1 nM) for 7 days. The experiment was repeated three times.

Animals and implantation of tumor cells

Male athymic BALB/c nude mice, 8 weeks old, were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA), and housed in a controlled environment. The use of animals was approved by the animal ethical committee in Gothenburg.

For subcutaneous implantation, two million tumor cells suspended in equal volumes of medium and matrigel (BD Biosciences, Bedford, MA, USA) were inoculated in the flank of the mice. Castration or sham operation was performed via a scrotal incision under

anesthesia prior to cell injection. Orthotopic implantation of tumor cells was performed as described previously (Jennbacken *et al.* 2009). Briefly, one million tumor cells suspended in 7 μ l matrigel (BD Biosciences) were injected into the dorsolateral lobe of the prostate. In experiments involving castrated mice, castration was performed prior to cell injection via the abdominal incision. The animal experiments were discontinued after 9 weeks. Tumors were harvested, and one part was fixed in formalin for paraffin embedding and the other part was frozen in liquid nitrogen and stored at -80°C prior to protein and RNA preparation.

For the metastasis experiment, male SCID CB17 mice, 7 weeks old, were used (Charles River Laboratories International, Inc.). LNCaP-19 cells were implanted in the prostate in intact and castrated mice as described above. After 15 weeks, mice were killed and examined for metastasis. Primary tumors and macroscopic lymph node metastases were collected, and tissues were treated as described above.

Patient characteristics and tissue specimens

Formalin-fixed and paraffin-embedded tissues were obtained from patients with prostate cancer from the Department of Urology, Sahlgrenska University Hospital, Gothenburg, Sweden. The studies with human material were conducted with ethical approval of the local research ethical committee.

Transurethral resection of the prostate samples

Tissue specimens were obtained from 54 patients by transurethral resection of the prostate (TURP). Twenty-eight patients had TURP-diagnosed untreated prostate cancer in stage T1b, and 25 patients had recurrent castration-resistant prostate cancer after ADT. One of the patients in the castration-resistant group underwent TURP at two different occasions, and therefore, the tissue specimens in this group were 26. In the hormone-naïve T1b group, mean age was 77 years (range 60–90) and mean Gleason score was 6.6 (range 5–9). In the castration-resistant group, mean age was 79 years (range 65–88) and mean Gleason score was 9.1 (range 7–10).

Biopsy samples

Tissue specimens were obtained from 28 patients by needle biopsies. Biopsies were obtained sequentially during prostate cancer progression, and prostate-specific antigen (PSA) was measured in serum at the same time points. Biopsy 1 was sampled at the time of prostate cancer diagnosis prior to ADT. Biopsy 2 was sampled ~ 3 months after initiation of ADT. The ADT

consisted of medical or surgical castration. Biopsy 3 was sampled when the tumor relapsed as indicated by a rise in PSA or when the patients had symptomatic progression. The third biopsy was sampled from 10 of the 28 patients. Mean Gleason scores in the groups were 7.3, 7.2 and 8.1 respectively. Mean age at the time of diagnosis was 73 years (range 55–86).

RNA preparation and real-time RT-PCR

Total RNA from tumors was extracted as described previously (Jennbacken *et al.* 2009), and total RNA from cultured cells was extracted using the RNeasy Plus Mini Kit (Qiagen GmbH). RNA quality and concentration were measured using a bioanalyzer (Agilent 2100, Agilent Technologies Inc., Santa Clara, CA, USA) and nanodrop respectively. The RNA was reverse transcribed into cDNA as described previously (Tesan *et al.* 2008).

Real-time RT-PCR was performed using the ABI Prism 7500 Fast Sequence Detector (Applied Biosystems, Applied Biosystems Corporation, Foster City, CA, USA). PCR primers and TaqMan MGB probes targeting N-cadherin (Hs00983062_m1), E-cadherin (Hs00170423_m1), Vimentin (Hs00958116_m1), Snail (Hs00195591_m1), Slug (Hs00161904_m1), Twist1 (Hs00361186_m1), ZEB1 (Hs00232783_m1), and endogenous control 18S rRNA (Hs99999901) were purchased as TaqMan Gene Expression Assays (Applied Biosystems). PCR parameters were according to the manufacturer's protocol, and the $\Delta\Delta C_t$ method was used for relative mRNA quantification. PCRs for target genes and control were performed in duplicates for all samples, and were repeated twice.

Protein preparation and western blotting

Total protein was prepared from tumor tissue by homogenization and sonication in the presence of protease inhibitors (Complete Mini, Roche Diagnostics GmbH). Protein concentrations were measured using the BCA Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Western blotting was performed as described previously (Jennbacken *et al.* 2006). Primary antibodies used were N-cadherin (1/500 #610921, BD Biosciences) and E-cadherin (1/1000, #610182, BD Biosciences). Actin (1:2000, A2066, Sigma-Aldrich) was used as the loading control. As positive controls for the antibodies, rat brain was used for N-cadherin and OVCAR-3 cell lysate was used for E-cadherin (Jennbacken *et al.* 2006). Chemiluminescent signals were visualized using a LAS-4000 CCD camera (Fujifilm, Tokyo, Japan).

Immunohistochemistry

Tissue sections, 4 µm, were deparaffinized and rehydrated. Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) as described previously (Jennbacken *et al.* 2006). Primary antibodies were against N-cadherin (1/100 for the TURP specimens and 1/150 for the biopsies, #M3613, DAKO, Glostrup, Denmark), E-cadherin (1/300 for the xenografts and 1/10 000 for the human biopsies, #610182, BD Biosciences), chromogranin A (1/500 #Ab-1 LK2H10, Neomarkers, Freemont, CA, USA), and neuron-specific enolase (NSE; 1/5000 #Ab-1, clone E27, Neomarkers). Negative controls were performed by omitting the primary antibodies. Pancreas was used as the positive control for chromogranin A and NSE.

Evaluation of N-cadherin and E-cadherin immunostaining in human samples

N-cadherin was evaluated as the proportion of positive tumors cells in the whole section. There was no large variation in staining intensity between tumors, and therefore, intensity was not taken into consideration. Proportion of positive cells was scored as 0, no positive cells; 1, <25% positive cells; 2, 25–50% positive cells; 3, 50–75% positive cells; and 4, >75% positive cells.

For E-cadherin, intensity varied considerably between tumors, and E-cadherin was therefore evaluated by combining the proportion of positive tumor cells with staining intensity. Proportion of positive cells was scored as 0, no positive cells; 1, <1/3 positive cells; 2, 1/3–2/3 positive cells; and 3, >2/3 positive cells. Intensity was scored as 0, no detectable signal; 1, weak staining; 2, moderate staining; and 3, strong staining. The total score was obtained by multiplying the intensity score with the positive number score. Sections were evaluated in a blinded fashion in 200× magnification using a light microscope.

Statistical analysis

Mann–Whitney *U* test was used to analyze the differences between independent groups, and Wilcoxon signed-rank test was used to analyze the differences between paired groups (i.e. human biopsy material). Spearman's rank correlation test was used to analyze the correlations. Comparison of the proportion of positive N-cadherin cases in different groups was done with χ^2 test. Data are presented as mean $\pm$ s.e.m. A *P* value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 16.0 software for Windows (Chicago, IL, USA).

Results

N-cadherin increased by castration in AI LNCaP-19 xenografts

In this study, we investigated whether N-cadherin was affected by androgen deprivation, which is commonly used to treat advanced stage prostate cancer. We found that castration increased N-cadherin protein levels in the AI LNCaP-19 tumors. The negative regulation of N-cadherin by androgens was observed in both subcutaneous and orthotopic tumors (Fig. 1A and B). The difference was also observed at mRNA levels (data not shown). The AI PC-3 tumors, which in contrast to LNCaP-19 tumors do not express AR, also displayed N-cadherin, but no androgen regulation was found. In contrast, N-cadherin was absent in LNCaP tumors, and the expression was not induced by castration (Fig. 1A and B).

Inoculation of LNCaP-19 cells into the prostate of SCID mice resulted in the formation of macroscopic metastases to the lymph nodes (sacral, lumbar, and renal). Similarly to the primary tumors, higher expression of N-cadherin was detected in metastases from castrated mice than in those from intact mice.

Figure 1 N-cadherin expression in tumor xenografts analyzed by western blotting. (A) Subcutaneous and (B) orthotopic LNCaP, LNCaP-19, and PC-3 tumors grown in intact or castrated mice. Images show two representative samples of at least three in each group. (C) N-cadherin expression in orthotopic LNCaP-19 primary tumors and lymph node metastases from intact and castrated mice. Actin was used as a loading control. N-cadherin was detected at a molecular size of 130 kDa, and actin at 42 kDa. Int, tumors from intact mice; cast, tumors from castrated mice; p, primary tumor; m, lymph node metastasis.

No major differences in N-cadherin expression between primary tumors and metastases in the intact or castrated group were found (Fig. 1C).

N-cadherin increased by androgen deprivation in AI LNCaP-19 cells *in vitro*

To investigate if androgens affected N-cadherin directly or via the surrounding tumor environment, *in vitro* experiments were performed. Corresponding to the *in vivo* experiments, androgen deprivation significantly increased N-cadherin mRNA expression in the LNCaP-19 cells. The expression increased about three times in comparison to cultures in the presence of androgens (0.1 and 1 nM R1881). Furthermore, the upregulation of N-cadherin in LNCaP-19 cells could be reversed if synthetic androgens were added, and there was a negative association between N-cadherin expression and androgen concentration (Fig. 2).

In PC-3 cells, N-cadherin mRNA could be detected, but its expression was not altered by removal of androgens. The LNCaP cells did not express detectable levels of N-cadherin mRNA in cultures, neither in the presence or in the absence of androgen (Fig. 2).

E-cadherin decreased after castration in orthotopic AI LNCaP-19 xenografts

Increased levels of N-cadherin are often accompanied by a concomitant decrease in E-cadherin. Therefore, we investigated if E-cadherin decreased by androgen

Figure 2 N-cadherin expression *in vitro* was analyzed by real-time RT-PCR. Cells were cultured in the presence of androgens (0.1 or 1 nM R1881) or in the absence of androgens (0 nM R1881) for 10 days, and then, the N-cadherin mRNA was analyzed (indicated as day 10 in the figure). To investigate if the increase in N-cadherin by androgen deprivation could be reversed, a proportion of the LNCaP-19 cells cultured in the absence of androgens were continued in culture and androgens were added (0.1 or 1 nM). After 7 days, N-cadherin mRNA was analyzed (indicated as day 10+7 in the figure). The $\Delta\Delta C_t$ method was used to calculate relative expression, and 18S was used as endogenous control. Results are presented as mean $\pm$ S.E.M. ($n=3$).

Figure 3 Expression of E-cadherin protein in LNCaP-19 tumor xenografts. Western blot images of (A) subcutaneous and (B) orthotopic LNCaP-19 tumors grown in intact or castrated mice. Images show two representative samples of five in each group. Actin was used as a loading control. E-cadherin was detected at a molecular size of 120 kDa, and actin at 42 kDa. The downregulation of E-cadherin in orthotopic tumors from castrated mice was confirmed by immunohistochemistry. Immunostaining was localized in the cell membrane. (C) Tumors from intact mice showed a homogeneous positive E-cadherin reaction from most tumor cells, while E-cadherin expression in (D) tumors from castrated mice showed a more scattered staining pattern (arrows) and were not present on every cell. Magnification 400 $\times$.

deprivation in LNCaP-19 cells. In subcutaneous LNCaP-19 tumors, castration did not alter the E-cadherin levels (Fig. 3A). In contrast, castration reduced E-cadherin levels in the orthotopic tumors (Fig. 3B). This result was confirmed by immunohistochemistry. E-cadherin was uniformly distributed at

the surface of the tumor cells in orthotopic LNCaP-19 tumors from intact mice (Fig. 3C). However, in the tumors from castrated mice, the staining was weaker, more scattered, and not present on every cell (Fig. 3D).

Androgens did not downregulate E-cadherin in the LNCaP-19 cells *in vitro*, showing the importance of the prostatic environment for this process (data not shown).

Expression of transcription factors related to EMT and neuroendocrine markers

To address if upregulation of N-cadherin was related to EMT, expression of the mesenchymal marker Vimentin and of transcription factors known to activate N-cadherin during induction of EMT was investigated with real-time PCR in the animal model. However, there were no apparent differences in the mRNA levels of Twist1, Snail, Slug, ZEB1, or Vimentin that could explain the increased levels of N-cadherin observed after castration (data not shown). To investigate whether N-cadherin was part of the neuroendocrine (NE) differentiation often seen in prostate cancer after ADT, markers for NE were studied by immunohistochemistry in the animal model. No association between N-cadherin and chromogranin A or NSE was detected (data not shown). These results indicate that the upregulation of N-cadherin was not associated with EMT or NE.

N-cadherin increased in castration-resistant prostate cancer and was associated with metastasis

N-cadherin was evaluated in hormone-naïve and castration-resistant TURP prostate cancer samples by immunohistochemistry. N-cadherin staining was mainly observed at the plasma membrane of the tumor cells, but some areas also showed cytoplasmic staining (Fig. 4A). Benign prostate epithelial cells were negative (Fig. 4B). In addition to the tumor cells, macrophages showed positive reactions and

Figure 4 Expression of N-cadherin in human prostate cancer TURP specimens was evaluated by immunohistochemistry. A scoring system for the proportion of positive tumor cells in the section was used. (A) N-cadherin was located in the plasma membrane of the cancer cells, and some areas also showed cytoplasmic staining. (B) Benign prostate epithelial cells were negative (arrow). Positive N-cadherin staining from cancer is indicated by an arrowhead. Magnification 200 $\times$. Magnification in the inset is at 400 $\times$. Mean N-cadherin score in (C) hormone-naïve (HN) and castration-resistant (CR) tumors and (D) in prostate tumors from patients with established metastases (M1) and patients without known metastases (M0). Data are presented as mean $\pm$ S.E.M. * $P < 0.05$ versus HN/M0.

served as convenient internal positive controls. In the hormone-naïve group, 50% (14/28) of the tumors were positive for N-cadherin compared with 81% (21/26) in the castration-resistant group ($P=0.018$). In addition, the castration-resistant tumors had a significantly higher N-cadherin score than the hormone-naïve tumors (1.42 vs 0.79 respectively, $P=0.023$; Fig. 4C). There was also a positive correlation between the N-cadherin score and the Gleason score (Spearman's correlation coefficient = 0.485, $P<0.001$; data not shown). Furthermore, the castration-resistant group was divided into two groups based on the metastasis status of the patients. In tumors from patients with established metastases, 93% (14/15) were N-cadherin positive compared with 56% (5/9) of the tumors from patients without known metastases ($P=0.027$). Tumors from patients with metastases (M1) also displayed a significantly higher N-cadherin score compared with tumors from patients without metastases (M0; 1.73 vs 0.78 respectively, $P=0.048$; Fig. 4D).

N-cadherin increased after initiation of ADT in human prostate cancer

To evaluate the direct effect of ADT on N-cadherin expression in human prostate cancer, N-cadherin was studied in prostate biopsies taken sequentially during tumor progression. N-cadherin was found in 46% (13/28) of the biopsies sampled at diagnosis (biopsy 1), and the mean N-cadherin score was 0.7. Three months after initiation of ADT (biopsy 2), 82% (23/28) of the biopsies displayed N-cadherin staining, and the mean score had increased to 1.9, which was statistically significant from that of biopsy 1 ($P<0.001$). In the relapsed castration-resistant group (biopsy 3), 70% (7/10) of the biopsies were positive, and the mean N-cadherin score was 2.4, which was also statistically significant from that of biopsy 1 ($P=0.038$; Fig. 5A).

Immunostaining of E-cadherin was also evaluated in the biopsy material. E-cadherin was located at the cell membrane of benign epithelial cells and tumor cells. A majority of the cases in each group were positive for E-cadherin, and there was no major difference in the E-cadherin score between biopsies 1, 2, and 3 (4.1 vs 3.1 vs 4.7; Fig. 5B).

The patients' PSA value was measured at the time when biopsies were collected. The PSA values declined tenfold after initiation of ADT, showing that the tumors responded to the treatment. At tumor relapse, the PSA value increased approximately fourfold compared with the low level at the time of biopsy 2 (Fig. 5C).

Figure 5 Expression of N-cadherin and E-cadherin in human prostate cancer biopsies was evaluated by immunohistochemistry. For N-cadherin, a scoring system for the proportion of positive tumor cells in the section was used. For E-cadherin, proportion of positive tumor cells was combined with staining intensity. Biopsies were collected sequentially from untreated, hormone-naïve tumors at the time of prostate cancer diagnosis (biopsy 1) and 3 months after initiation of ADT (biopsy 2), and from the recurrent castration-resistant tumors (biopsy 3). (A) The N-cadherin score and (B) the E-cadherin score in the tumor biopsies. (C) Relative PSA values at the time when the prostate cancer biopsies were taken. Data are presented as mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus biopsy 1.

Discussion

In this study, we showed that N-cadherin was induced by androgen deprivation in experimental AI prostate cancer as well as in human prostate tumors. Moreover, expression of N-cadherin in human samples was associated with metastasis and Gleason score.

To our knowledge, the downregulation of N-cadherin by androgens in prostate cancer has not been reported previously. In contrast, androgenic

upregulation of N-cadherin has been described earlier in motoneurons (Monks & Watson 2001). The N-cadherin gene contains a cluster of androgen response elements in its intron 1 (Takayama et al. 2007), and it is possible that the repressed N-cadherin gene expression is attributed to a direct inhibition by binding of activated AR to this site. Even though the main effect by androgens on prostate cells through the AR is stimulatory, inducing proliferation and anti-apoptotic responses, negative regulation by androgens has also been reported (Wikstrom et al. 1999, Li et al. 2005, Terry et al. 2009). In this study, expression of N-cadherin was found in the AI cell lines LNCaP-19 and PC-3. However, it was only in the AR-positive LNCaP-19 cell line that N-cadherin levels could be elevated further by androgen deprivation, thus demonstrating the importance of AR in the regulation of N-cadherin. Exactly how AR and androgens regulate N-cadherin is at present unknown, and further studies are needed to be able to address this issue.

In general, the regulation of E-cadherin and N-cadherin is believed to be tightly connected, resulting in a switch from one to another. In this study, we observed different E-cadherin responses to castration, depending on the location of the tumor. In the orthotopic LNCaP-19 tumors, the upregulation of N-cadherin after castration was accompanied with decreased E-cadherin levels, as expected. However, this response could not be observed by androgen deprivation *in vitro* or in subcutaneous LNCaP-19 tumors. The reduction in E-cadherin in the orthotopic tumors from castrated mice was therefore probably an indirect effect mediated by the surrounding prostatic environment. Furthermore, there was no obvious increase in the mesenchymal marker Vimentin or the transcription factors Twist, Snail, Slug, or ZEB1 in the animal model that accompanied the increased N-cadherin levels. The results presented here therefore indicate a distinct regulatory mechanism of N-cadherin, which is not connected to the EMT and E-cadherin regulatory mechanisms.

In this study, N-cadherin was more frequently expressed in castration-resistant tumors compared with early-stage T1b prostate tumors, which is not unexpected, since it has been reported that N-cadherin increases in poorly differentiated prostate cancer and correlates to Gleason grade (Bussemakers et al. 2000, Tomita et al. 2000, Jaggi et al. 2006). Moreover, a switch from E-cadherin to N-cadherin has been correlated to the progression of prostate cancer in hormone-naïve tumors, and expression of N-cadherin was associated with pelvic lymph node infiltration and shorter time to skeletal metastases

(Gravdal et al. 2007). Whether the increase in N-cadherin observed after initiation of ADT really leads to a poor outcome for the patients and if it influences the propensity for tumor cells to form metastases remain to be clarified. However, a possible clue could be that patients with established metastases in this study more frequently expressed N-cadherin than patients without known metastases. The finding that N-cadherin was increased already 3 months after initiation of ADT (biopsy 2) and not only in the relapsed castration-resistant cases (biopsy 3) indicates that it could be a direct consequence of androgen deprivation and not of transition into the castration-resistant stage. In the literature, N-cadherin is mostly described as a factor that promotes migration and metastasis of tumor cells. However, N-cadherin has also been reported to be involved in the survival of tumor cells, via induction of anti-apoptotic pathways (Li et al. 2001, Tran et al. 2002). Consequently, induction of N-cadherin after ADT can promote a survival advantage for tumor cells at low androgen levels.

ADT is the standard treatment for patients with locally advanced or metastatic prostate cancer. The benefits for the patients are indisputable, and it has even been suggested that ADT should be initiated in an early phase of the disease to extend patient survival (Anderson 1999). However, conflicting results are reported in the literature regarding the effects of androgen deprivation on the cellular level. Disruption of the androgen signaling pathway by ADT may result in the deregulation of the cell control, which could contribute to the carcinogenic process. Early initiation of ADT might therefore speed the development of castration-resistant disease. In addition, it has been suggested that treatment with anti-androgens such as bicalutamide could induce alterations in the prostatic environment that promote emergence of castration-resistant tumors (Lee & Tenniswood 2004). Studies have shown that an intact androgen signaling pathway in prostate tumor cells decreased invasion and metastasis in animal models (Cinar et al. 2001, Niu et al. 2008). In contrast, there are also reports showing that testosterone signaling via AR promotes invasion of prostate tumor cells *in vitro* (Hara et al. 2008). Because of the contradicting data in the literature, further studies emphasizing the molecular effects of androgen deprivation on prostate cancer cells are clearly needed.

In the present study, we have shown that the proinvasive factor N-cadherin was increased by androgen deprivation, and correspondingly, there are other reports in the literature supporting the possibility that androgen deprivation could induce the metastatic machinery in the cells. Nestin, which is an intermediate

filament protein that has a role in metastasis of tumor cells, and the lymphatic growth factor vascular endothelial growth factor C are induced in response to androgen withdrawal (Li *et al.* 2005, Kleeberger *et al.* 2007, Rinaldo *et al.* 2007). Recently, there was also a report of augmented levels of class III β -tubulin after androgen ablation and in castration-resistant prostate tumors, which could explain resistance to therapy with taxanes in this disease stage (Terry *et al.* 2009). With these results in hand, it is tempting to speculate if ADT induces molecular alterations in the tumor cells which will give them an advantage in forming metastases in the relapsed castration-resistant stage.

Increased levels of N-cadherin in poorly differentiated and castration-resistant prostate cancer specimens open the possibility of using N-cadherin antagonists as the second-line therapy for castration-resistant prostate cancer. At present, an anti-N-cadherin peptide, ADH-1 (Exherin), is being evaluated in clinical trials against N-cadherin-expressing tumors. Our results warrant further investigations of the role of N-cadherin in aggressive prostate cancer. Targeted therapy using an N-cadherin antagonist in combination with chemotherapy or other targeted therapies could also be a novel approach for treating metastatic and castration-resistant prostate cancer.

Taken together, this study demonstrates that N-cadherin, a potentially important factor for the formation of metastases, was induced by androgen deprivation in experimental AI prostate cancer and in human prostate cancer. Furthermore, expression of N-cadherin showed a positive association with Gleason score and metastases. The results indicate that ADT may directly influence the prostate cancer cells to acquire properties associated with the metastatic phenotype observed in castration-resistant prostate cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Swedish Cancer Society, Sahlgrenska University Hospital, Swedish Johanniterhjälpen, Swedish Medical Society, Research Foundations of af Jochnick, C & S Hagströmer, A Gabriellsson, M Bergvall, Å Wiberg, L Hierta, A & B Björnsson, W & M Lundgren, M & G Ågren and H Fries.

Acknowledgements

The authors would like to thank Anita Fae and Karina Jernsand for excellent technical assistance during animal experiments and laboratory work. They also thank Ulric Pedersen for help with the illustrations.

References

- Anderson JB 1999 Early versus deferred hormone therapy. *European Urology* **36** (Supplement 2) 9–13.
- Augustine CK, Yoshimoto Y, Gupta M, Zipfel PA, Selim MA, Febbo P, Pendergast AM, Peters WP & Tyler DS 2008 Targeting N-cadherin enhances antitumor activity of cytotoxic therapies in melanoma treatment. *Cancer Research* **68** 3777–3784.
- Beasley GM, McMahon N, Sanders G, Augustine CK, Selim MA, Peterson B, Norris R, Peters WP, Ross MI & Tyler DS 2009 A phase 1 study of systemic ADH-1 in combination with melphalan via isolated limb infusion in patients with locally advanced in-transit malignant melanoma. *Cancer* **115** 4766–4774.
- Bussemakers MJ, Van Bokhoven A, Tomita K, Jansen CF & Schalken JA 2000 Complex cadherin expression in human prostate cancer cells. *International Journal of Cancer* **85** 446–450.
- Chambers AF, Groom AC & MacDonald IC 2002 Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews. Cancer* **2** 563–572.
- Chu YS, Thomas WA, Eder O, Pincet F, Perez E, Thierry JP & Dufour S 2004 Force measurements in E-cadherin-mediated cell doublets reveal rapid adhesion strengthened by actin cytoskeleton remodeling through Rac and Cdc42. *Journal of Cell Biology* **167** 1183–1194.
- Cinar B, Koeneman KS, Edlund M, Prins GS, Zhou HE & Chung LW 2001 Androgen receptor mediates the reduced tumor growth, enhanced androgen responsiveness, and selected target gene transactivation in a human prostate cancer cell line. *Cancer Research* **61** 7310–7317.
- Damber JE & Aus G 2008 Prostate cancer. *Lancet* **371** 1710–1721.
- Derycke LD & Bracke ME 2004 N-cadherin in the spotlight of cell–cell adhesion, differentiation, embryogenesis, invasion and signalling. *International Journal of Developmental Biology* **48** 463–476.
- Derycke L, De Wever O, Stove V, Vanhooeck B, Delanghe J, Depypere H & Bracke M 2006a Soluble N-cadherin in human biological fluids. *International Journal of Cancer* **119** 2895–2900.
- Derycke L, Morbidelli L, Ziche M, De Wever O, Bracke M & Van Aken E 2006b Soluble N-cadherin fragment promotes angiogenesis. *Clinical and Experimental Metastasis* **23** 187–201.
- Giehle K & Menke A 2008 Microenvironmental regulation of E-cadherin-mediated adherens junctions. *Frontiers in Bioscience* **13** 3975–3985.
- Gravdal K, Halvorsen OJ, Haukaas SA & Akslen LA 2007 A switch from E-cadherin to N-cadherin expression

- indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. *Clinical Cancer Research* **13** 7003–7011.
- Gregory CW, He B, Johnson RT, Ford OH, Mohler JL, French FS & Wilson EM 2001 A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Research* **61** 4315–4319.
- Gustavsson H, Welen K & Damber JE 2005 Transition of an androgen-dependent human prostate cancer cell line into an androgen-independent subline is associated with increased angiogenesis. *Prostate* **62** 364–373.
- Hara T, Miyazaki H, Lee A, Tran CP & Reiter RE 2008 Androgen receptor and invasion in prostate cancer. *Cancer Research* **68** 1128–1135.
- Hatta K, Takagi S, Fujisawa H & Takeichi M 1987 Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Developmental Biology* **120** 215–227.
- Hazan RB, Kang L, Whooley BP & Borgen PI 1997 N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. *Cell Adhesion and Communication* **4** 399–411.
- Hazan RB, Phillips GR, Qiao RF, Norton L & Aaronson SA 2000 Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *Journal of Cell Biology* **148** 779–790.
- Jaggi M, Nazemi T, Abrahams NA, Baker JJ, Galich A, Smith LM & Balaji KC 2006 N-cadherin switching occurs in high Gleason grade prostate cancer. *Prostate* **66** 193–199.
- Jennbacken K, Gustavsson H, Welen K, Vallbo C & Damber JE 2006 Prostate cancer progression into androgen independency is associated with alterations in cell adhesion and invasivity. *Prostate* **66** 1631–1640.
- Jennbacken K, Gustavsson H, Tesan T, Horn M, Vallbo C, Welen K & Damber JE 2009 The prostatic environment suppresses growth of androgen-independent prostate cancer xenografts: an effect influenced by testosterone. *Prostate* **69** 1164–1175.
- Kleeberger W, Bova GS, Nielsen ME, Herawi M, Chuang AY, Epstein JI & Berman DM 2007 Roles for the stem cell associated intermediate filament Nestin in prostate cancer migration and metastasis. *Cancer Research* **67** 9199–9206.
- Lee EC & Tenniswood MP 2004 Emergence of metastatic hormone-refractory disease in prostate cancer after anti-androgen therapy. *Journal of Cellular Biochemistry* **91** 662–670.
- Li G, Satyamoorthy K & Herlyn M 2001 N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Research* **61** 3819–3825.
- Li J, Wang E, Rinaldo F & Datta K 2005 Upregulation of VEGF-C by androgen depletion: the involvement of IGF-IR-FOXO pathway. *Oncogene* **24** 5510–5520.
- Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL & Visakorpi T 2001 Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Research* **61** 3550–3555.
- Maeda M, Johnson KR & Wheelock MJ 2005 Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. *Journal of Cell Science* **118** 873–887.
- Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A *et al.* 2000 Androgen receptor mutations in prostate cancer. *Cancer Research* **60** 944–949.
- Monks DA & Watson NV 2001 N-cadherin expression in motoneurons is directly regulated by androgens: a genetic mosaic analysis in rats. *Brain Research* **895** 73–79.
- Niu Y, Altuwaijri S, Lai KP, Wu CT, Ricke WA, Messing EM, Yao J, Yeh S & Chang C 2008 Androgen receptor is a tumor suppressor and proliferator in prostate cancer. *PNAS* **105** 12182–12187.
- Perotti A, Sessa C, Mancuso A, Noberasco C, Cresta S, Locatelli A, Carcangiu ML, Passera K, Braghetta A, Scaramuzza D *et al.* 2009 Clinical and pharmacological phase I evaluation of ExherinTM (ADH-1), a selective anti-N-cadherin peptide in patients with N-cadherin-expressing solid tumours. *Annals of Oncology* **20** 741–745.
- Rinaldo F, Li J, Wang E, Muters M & Datta K 2007 RalA regulates vascular endothelial growth factor-C (VEGF-C) synthesis in prostate cancer cells during androgen ablation. *Oncogene* **26** 1731–1738.
- Salomon D, Ayalon O, Patel-King R, Hynes RO & Geiger B 1992 Extrajunctional distribution of N-cadherin in cultured human endothelial cells. *Journal of Cell Science* **102** 7–17.
- Sandig M, Voura EB, Kalnins VI & Siu CH 1997 Role of cadherins in the transendothelial migration of melanoma cells in culture. *Cell Motility and the Cytoskeleton* **38** 351–364.
- Shintani Y, Fukumoto Y, Chaika N, Grandgenett PM, Hollingsworth MA, Wheelock MJ & Johnson KR 2008 ADH-1 suppresses N-cadherin-dependent pancreatic cancer progression. *International Journal of Cancer* **122** 71–77.
- Suyama K, Shapiro I, Guttman M & Hazan RB 2002 A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell* **2** 301–314.
- Takayama K, Kaneshiro K, Tsutsumi S, Horie-Inoue K, Ikeda K, Urano T, Ijichi N, Ouchi Y, Shirahige K, Aburatani H *et al.* 2007 Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis. *Oncogene* **26** 4453–4463.
- Terry S, Ploussard G, Allory Y, Nicolaiew N, Boissiere-Michot F, Maille P, Kheuang L, Coppolani E,

- Ali A, Bibeau F *et al.* 2009 Increased expression of class III beta-tubulin in castration-resistant human prostate cancer. *British Journal of Cancer* **101** 951–956.
- Tesan T, Gustavsson H, Welen K & Damber JE 2008 Differential expression of angiopoietin-2 and vascular endothelial growth factor in androgen-independent prostate cancer models. *BJU International* **102** 1034–1039.
- Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ & Schalken JA 2000 Cadherin switching in human prostate cancer progression. *Cancer Research* **60** 3650–3654.
- Tran NL, Nagle RB, Cress AE & Heimark RL 1999 N-Cadherin expression in human prostate carcinoma cell lines. An epithelial–mesenchymal transformation mediating adhesion with stromal cells. *American Journal of Pathology* **155** 787–798.
- Tran NL, Adams DG, Vaillancourt RR & Heimark RL 2002 Signal transduction from N-cadherin increases Bcl-2. Regulation of the phosphatidylinositol 3-kinase/Akt pathway by homophilic adhesion and actin cytoskeletal organization. *Journal of Biological Chemistry* **277** 32905–32914.
- Wikstrom P, Westin P, Stattin P, Damber JE & Bergh A 1999 Early castration-induced upregulation of transforming growth factor beta1 and its receptors is associated with tumor cell apoptosis and a major decline in serum prostate-specific antigen in prostate cancer patients. *Prostate* **38** 268–277.
- Xie D, Gore C, Liu J, Pong RC, Mason R, Hao G, Long M, Kabbani W, Yu L, Zhang H *et al.* 2010 Role of DAB2IP in modulating epithelial-to-mesenchymal transition and prostate cancer metastasis. *PNAS* **107** 2485–2490.