Nectin-4: a new prognostic biomarker for efficient therapeutic targeting of primary and metastatic triple-negative breast cancer

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Background: Triple-negative breast cancers (TNBCs) are associated with a poor prognosis. In contrast to other molecular subtypes, they have no identified specific target and chemotherapy remains the only available systemic treatment. The adhesion molecule nectin-4 represents a new potential therapeutic target in different cancer models. Here, we have tested the prognostic value of nectin-4 expression and assessed the therapeutic efficiency of an anti-nectin 4 antibody drug conjugate (ADC) on localised and metastatic TNBC

Materials and methods: We analysed nectin-4/PVRL4 mRNA expression in 5673 invasive breast cancers and searched for correlations with clinicopathological features including metastasis-free survival (MFS). Immunohistochemistry was carried out in 61 TNBCs and in samples of primary TNBC Patient-Derived Xenografts (PDXs). An anti-nectin-4 antibody eligible for ADC was produced and tested in vitro and in vivo in localised and metastatic TNBC PDXs.

Results: High nectin-4/PVRL4 mRNA expression was associated with poor-prognosis features including the TN and basal subtypes. High PVRL4 mRNA expression showed independent negative prognostic value for MFS in multivariate analysis in TNBCs. Nectin-4 protein expression was not detected in adult healthy tissues including mammary tissue. Membranous protein expression was found in 62% of TNBCs, with strong correlation with mRNA expression. We developed an ADC (N41mab-vcMMAE) comprising a human anti-nectin-4 monoclonal antibody conjugated to monomethyl auristatin-E (MMAE). In vitro, this ADC bound to nectin-4 with high affinity and specificity and induced its internalisation as well as dose-dependent cytotoxicity on nectin-4-expressing breast cancer cell lines. In vivo, this ADC induced rapid, complete and durable responses on nectin-4-positive xenograft TNBC samples including primary tumours, metastatic lesions, and local relapses; efficiency was dependent on both the dose and the nectin-4 tumour expression level.

Conclusion: Nectin-4 is both a new promising prognostic biomarker and specific therapeutic target for ADC in the very limited armamentarium against TNBC.

Key words: ADC targeting, biomarker, breast cancer, nectin-4, survival, TNBC

Introduction

Triple-negative breast cancers (TNBCs) represent about 15–20% of breast cancers, and are characterised by the absence of expression of oestrogen and progesterone receptors and the absence of HER2 overexpression or gene amplification. They show overlapping genomic characteristics with basal BCs [1–3]. Patients with TNBC have a higher risk of early and visceral relapse than patients with non-TN breast cancer [4]. However, despite recent efforts to find alternative treatments such as hormone therapy and anti-
HER2 drugs for, respectively, hormone receptor-positive and HER2-positive breast cancers, there are no efficient targeted therapies for TNBCs and chemotherapy remains the only available systemic treatment [5]. A cell surface protein specifically expressed in TNBC malignant cells and not in normal breast would consequently represent an ideal molecular surface biomarker as well as a potential therapeutic target for antibody–drug conjugate (ADC).

ADCs are monoclonal antibodies conjugated to cytotoxic agents. Antibodies are specific to tumour cell-surface proteins, and thus, have tumour specificity and potency not achievable with traditional drugs [6]. Design of effective ADC for cancer therapy requires the identification of an appropriate target, a monoclonal antibody against the target, potent cytotoxic effectors, and conjugation of the monoclonal antibody to cytotoxic agents. Two ADCs recently received approval from the US Food and Drug Administration for treatment of CD30-positive lymphoma (brentuximab-vedotin) and HER2-positive breast cancer (trastuzumab-emtansine), and several new promising ADCs are now in late-phase clinical testing [6, 7].

PVRL4/Nectin-4 is involved in the formation and maintenance of adherens junctions in cooperation with cadherins [8–10]. Nectin-4 is a type I transmembrane cell adhesion molecule composed of three Ig-like domains (V–C–C type) in its extracellular region [8]. It is also a receptor for the measles virus, mediating its endocytosis [11]. Nectin-4 is expressed during foetal development, with expression declining in adult life by contrast with the extensive expression of the other nectins in adult tissues [8]. We and others have shown that nectin-4 is re-expressed as a tumour-associated antigen with pro-oncogenic properties in various carcinomas including breast cancer [12–17]. These features associated with its limited expression in the respective normal tissues, suggest that nectin-4 could be a therapeutic target for ADC in these cancers, as recently reported in bladder, breast and pancreatic cancers [17].

Here, we have analysed the mRNA and protein expression of nectin-4 in a series of ~5700 breast cancers and showed that nectin-4 is a cell surface biomarker more frequently overexpressed in TNBCs and basal breast cancers, and that mRNA expression is an independent poor-prognosis factor in TNBCs. Whole-genome microarray analysis [18] of a series of nectin-4-positive patient samples. The final pooled set included 5673 non-redundant, non-metastatic, non-inflammatory, primary, invasive breast cancers with PVRL4 mRNA expression and clinicopathological data available (supplementary Table S2, available at Annals of Oncology online).

For protein expression, we analysed a consecutive series of 61 previously untreated TNBC samples collected at the time of diagnosis from women treated at our Institute. Informed consent was obtained for each patient and the study was approved by our IRB (supplementary Table S3, available at Annals of Oncology online).

MDA-MB-231 and SUM190 breast cancer cell lines were used in this study. Detailed information is available in supplementary Methods (available at Annals of Oncology online).

Gene expression data analysis

Our own gene expression data set had been generated using Affymetrix U133 Plus 2.0 human microarrays (Affymetrix®, Santa Clara, CA) as described [18]. MIAME-compliant data are deposited in the GEO database (GSE31448). PVRL4 expression was measured by analysing different probe sets whose identity and specificity were verified using the NCBI program BLASTN 2.2.31+ (supplementary Table S4, available at Annals of Oncology online). Additional details are available in supplementary Methods (available at Annals of Oncology online).

Antibody production, immunohistochemistry, western-blot, ELISA and flow cytometry

Different nectin-4 antibodies were produced. Detailed information is available in supplementary Methods (available at Annals of Oncology online).

Production of ADC

The ADC was produced by Concertis (San Diego, CA). Conjugates were produced from purified mab1/N41mac monoclonal antibody. The linker used was the maleimidocaproyl-t-valine-t-citrulline-p-aminobenzyl alcohol p-nitrophenyl carbonate (MC-Val-Cit-PAB-PNP) covalently conjugated to monomethyl auristatin-E (MMAE). This cleavable linker was selected because it induced potent bystander killing. The drug-to-antibody ratio was 4.73.

Animal models

All experiments were done in agreement with the French Guidelines for animal handling and approved by local ethics committee (Agreement no. 01152-01). Additional details are available in supplementary Methods (available at Annals of Oncology online).

Statistical analysis

This section is detailed in supplementary Methods (available at Annals of Oncology online).

Results

Nectin-4/PVRL4 is expressed in TNBC and predicts poor prognosis

We examined PVRL4 expression in breast cancer. Whole-genome clustering of our 353-sample series showed that PVRL4 mRNA was in the ‘basal’ gene cluster and more consistently expressed in basal tumours (Figure 1A and B). We analysed nectin-4 protein expression by immunohistochemistry (IHC) in 61 TNBCs, 12 of them previously profiled using DNA microarrays. The monoclonal
The antibody used for this analysis, selected from our screening (see next paragraph), recognised the distal IgV-like domain of human nectin-4 and did not cross-react with the other human nectins or with mouse nectin-4 (supplementary Figure S1, available at Annals of Oncology online). Nectin-4 expression was detected at the plasma membrane. Based on the QuickScore (QS) semi-quantitative assessment, we distinguished a ‘nectin-4-high group’ (QS > 100) and a ‘nectin-4-low group’ (QS ≤ 100), representing 62% and 38% of TNBCs, respectively (Figure 1C). There was a positive correlation between mRNA and protein expression of nectin-4 (supplementary Figure S2, available at Annals of Oncology online; P = 0.002). Importantly, nectin-4 was detected neither in the normal mammary gland epithelium (Figure 1D) nor in 30 different adult normal tissues except the skin (supplementary...
Figure S3, available at *Annals of Oncology* online) [10]. These results established nectin-4 as both a new cell surface biomarker and a potential target for TNBCs.

We then analysed a pooled series of 5673 invasive breast cancers profiled using DNA microarrays and five different *PVRL4* probes 100% specific. High *PVRL4* expression was associated with poor-prognosis features, including both TN and basal subtypes (supplementary Table S5, available at *Annals of Oncology* online). *PVRL4* was highly expressed in 61% of TN samples and 62% of basal samples versus 47% of non-TN or non-basal samples. Metastasis-free survival (MFS) data were available for 1037 patients, including 613 without metastatic relapse (median follow-up, 83 months) and 424 with metastatic relapse (median time to relapse, 24 months). The 5-year MFS rate was 61% (95% CI, 0.51–0.74) in the ‘PVRL4-low’ groups, respectively (*P* = 0.014, log-rank test) (supplementary Figure S4, available at *Annals of Oncology* online). Prognostic univariate analysis per molecular subtype showed that high *PVRL4* expression was associated with shorter MFS (*P* = 0.014, log-rank test) (Figure 1E). In multivariate analysis (Table 1), high *PVRL4* expression remained significant (*P* = 0.039, Wald test; HR = 1.53 [1.02–2.30]). Altogether, these results indicated that *PVRL4* nectin-4 is commonly expressed in TN or basal breast cancers, not expressed in the normal breast, and is an independent predictor of shorter MFS in TNBCs.

### ADC-based targeting of nectin-4 in vitro

We tested six monoclonal antibodies (mAbs) directed against the IgV-like distal domain of nectin-4 to isolate a mAb able to induce internalisation. MAbs were evaluated for EC50, maximum binding capacity, cell surface internalisation and cytotoxicity (supplementary Figure S5, available at *Annals of Oncology* online). Internalisation was tested using ectopically expressed Flag-tagged nectin-4 expressed in MDA-MB-231 cells and FITC-labelled anti-Flag antibody (M2, Sigma-Aldrich, St. Louis, MO) to quantify residual surface nectin-4. Mab1 was the most efficient antibody. It induced a 60% decrease of cell surface nectin-4 in 24 h and a 60% cell growth inhibition after incubation with a goat anti-mouse monoclonal antibody conjugated to saporin (mab-ZAP kit, ATS-bio, San Diego, CA). Internalisation and cytotoxicity were positively correlated (*R*² = 0.96). Mab1 did not affect cell viability in vitro and tumour cell growth in vivo (data not shown). Mab1 was then conjugated to monomethyl auristatin-E (MMAE) via a cleavable valine-citrulline (vc) di-peptide linker (thereafter called N41mab-vcMMAE, ADC) to produce an ADC, which was then tested in vitro for specificity and efficacy on selected breast cancer cell lines. MDA-MB-231 cells, which express nectin-1, nectin-2, and nectin-3, but not nectin-4, were not sensitive to the ADC. However, ectopic expression of nectin-4 conferred sensitivity with an IC50 = 2 ng/ml (supplementary Figure S6A, available at *Annals of Oncology* online). SUM190 cells, which express endogenous nectin-4, were sensitive with an IC50 = 4 ng/ml (supplementary Figure S6B, available at *Annals of Oncology* online). These data showed the specificity and the efficacy of N41mab-vcMMAE.

### ADC-based targeting of nectin-4 in vivo

Activity of our ADC was tested in three in vitro models of TNBC developed in immunocompromised NSG mice. First, mice xenografted with SUM190 cells were treated with two successive i.v. doses of N41mab-vcMMAE (Figure 2A). These doses were not toxic for mice (supplementary Figure S7, available at *Annals of Oncology* online). N41mab-vcMMAE induced a rapid (4 days) and complete tumour regression, the duration of which was dose-dependent and lasted up to 40 days at the 10 mg/kg dose (Figure 2A). Interestingly, after relapse, tumours still kept their sensitivity to the ADC with tumour regression similar to that obtained after the first treatment, at least over a 6-months period (Figure 2B).

Second, we used patient-derived xenografts (PDX) of nine primary TNBCs. These pre-clinical models recapitulate breast cancer physiopathology [19]. Localisation and levels of nectin-4 expression in PDX were similar to that found in TNBC patients (supplementary Figure S8, available at *Annals of Oncology* online). Nectin-4 expression was prominently found at the plasma membrane in seven of nine PDX (QS > 100). TNBC PDX mice

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**Table 1. Univariate and multivariate prognostic analyses in the TNBC population**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Univariate</th>
<th>Multivariate</th>
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<tbody>
<tr>
<td></td>
<td><em>n</em></td>
<td>HR [95% CI]</td>
</tr>
<tr>
<td>Age, &gt;50 versus ≤50</td>
<td>204</td>
<td>1.13 [0.68–1.89]</td>
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<tr>
<td>Pathological type, lobular versus ductal</td>
<td>128</td>
<td>1.49 [0.36–6.24]</td>
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<tr>
<td>Pathological type, mixed versus ductal</td>
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<td></td>
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<tr>
<td>Pathological type, other versus IDC</td>
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<td></td>
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<tr>
<td>Pathological tumour size (pT), pT2–3 versus pT1</td>
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<td>1.39 [0.56–3.41]</td>
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<td>Pathological lymph node status (pN), positive versus negative</td>
<td>202</td>
<td>0.75 [0.49–1.15]</td>
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<tr>
<td>Pathological grade, 3 versus 1–2</td>
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<td>1.42 [0.72–2.78]</td>
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<tr>
<td>Immune response GES, poor versus good</td>
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<td>1.82 [1.19–2.80]</td>
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<tr>
<td>LCK GES, poor versus good</td>
<td>290</td>
<td>1.92 [1.34–2.76]</td>
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<tr>
<td>Kinase immune GES, poor versus good</td>
<td>290</td>
<td>1.82 [1.23–2.63]</td>
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<tr>
<td>PVRL4/nectin-4 mRNA expression, high versus low</td>
<td>290</td>
<td>1.65 [1.10–2.47]</td>
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Figure 2. In vivo efficacy of the ADC N41mab-vCMMAE in TNBC models. (A) SUM190 cells grown orthotopically as tumours in NSG mice show dose-dependent regression after a single treatment with ADC at the indicated doses (2 i.v. injections (arrows) at 4-days interval). (B) Similar to (A). First arrows: first treatment by 2 i.v. injections at 10 mg/kg; second and third arrows: same treatment at second and third relapses. Tumour regression kinetic after relapse is similar to regression observed after the first treatment. (C–F) TN patient-derived tumour grown orthotopically in NSG mice and treated with ADC at 10 mg/kg (2 i.v. injections at 4-days interval). (C) PDX400 (QS = 3/C2 100%); (D) PDX317 (QS = 2/C2 70%); (E) PDX348 (QS = 2/C2 50%); (F) PDX434 (QS = 1/C2 10%). A bystander effect, described for cleavable linkers, may account for the inhibition observed for T317 and T348. (G) Docetaxel treatment (10 mg/kg) of PDX348. Arrows indicate i.v. injection. Error bars represent mean ± SEM.
with different QS were treated with two successive i.v. doses of ADC. The degree of clinical response was roughly correlated with nectin-4 expression level: a rapid and complete tumour burden regression (and durable up to 35 days) was observed for PDX400 (QS = 300) and PDX 317 (QS = 140), to a lesser extent for PDX348 (QS = 100) (Figure 2C–E), but not observed for PDX343 (QS = 10) (Figure 2F). In contrast, treatment of PDX 348—sensitive to our ADC—by docetaxel (3 times 10 mg/kg i.p.) was ineffective (Figure 2G).

Third, to evaluate ADC treatment efficacy in a more advanced disease, we treated PDX400 and PDX317 mice developing spontaneous metastatic lesions from primary tumours. In both cases, treatment with two successive i.v. doses of ADC led to a rapid reduction and complete disappearance of all metastatic lesions observed by luminescence analysis (Figure 3). Metastatic recurrences were detected at days 42 and 57 for PDX400, and still not observed for PDX317 (ongoing experiment: day 245) (data not shown). These results showed that N4mab1-vcMMAE had a marked anti-tumour activity in both primary and metastatic nectin-4-expressing TNBCs.

**Discussion**

Nectin-4 has been reported as a new tumour antigen for different carcinomas [12–17]. Here, we have analysed its mRNA and protein expression in a series of ~5700 breast cancers and showed that nectin-4 is a cell surface biomarker more frequently overexpressed in TNBCs and basal breast cancers, and that high mRNA expression is an independent poor-prognosis factor for MFS in TNBCs. Then, we developed an anti-nectin-4 ADC and showed its therapeutical efficiency on localised and metastatic TNBC both *in vitro* and *in vivo*, with rapid and long-lasting regression in nectin-4-positive samples. To our knowledge, this work is the first description of a cell surface biomarker eligible for the systemic treatment of TNBCs.

We showed that nectin-4 is a cell surface biomarker more frequently overexpressed in TNBCs and basal breast cancers. Its protein expression, analysed by IHC using our antibody, was detected at the plasma membrane and positively correlated with mRNA expression. High-protein expression and high mRNA expression were found in the same proportion of cases, nearly two-thirds of TNBCs. This protein expression pattern was confirmed using four other monoclonal antibodies recognising different epitopes in the nectin-4 ectodomain (supplementary Figure S9, available at *Annals of Oncology* online). Importantly, nectin-4 protein was not detected in the normal mammary gland epithelium and 30 other different adult normal tissues except the skin. These results confirmed and extended our previous work done within a limited number of samples [12] (Figure 1). To our knowledge, nectin-4 expression has been studied in clinical breast cancer samples in three other studies using IHC in two series of 197 [20] and 654 [17] cases and using IHC in a series of imprint smears of 140 cases [21]. Two studies used the same commercial polyclonal antibody [20, 21]. We tested this antibody on nectin-4 mRNA negative TNBCs and found a cytoplasmic and a nuclear staining by IHC (data not shown). This polyclonal antibody recognised nectin-4 by western-blot but, in contrast to N41mab, gave a high background signal on nectin-4 mRNA negative MDA-MB-231 cells.
Nectin-4 presents two interesting properties: an inducible and efficient internalisation, which is hijacked by the measles virus, and a limited expression in healthy tissues. Together, these properties make nectin-4 an attractive target for the development of immune-base therapies especially for TNBCs for which no specific cell surface antigen has been identified so far [22]. ADC directed against nectin-4 has been recently shown efficient in mice models subcutaneously xenografted with cell lines [17]. Based on in vitro assessment of EC50, maximum binding capacity, cell surface internalisation and cytotoxicity, we retained the Mab1 for ADC approach. Mab1 was then conjugated to monomethyl auristatin-E (MMAE) via a cleavable linker to produce an ADC, thereafter called N41mab-vcMMAE. This ADC was validated in vitro for efficiency and specificity.

We then showed its in vivo activity in models of TNBC developed in immunocomprised NSG mice. The model based on the SUM190 cell line exhibited rapid and complete response, the duration of which was prolonged and dependent on the dose; furthermore, the relapses remained similarly sensitive to our ADC over the 6-months period tested. We also took advantage of our collection of primary TNBC PDx phenotypically close to the physiopathology of TNBCs [19]. In these models, patient tumours develop in the mouse mammary gland and spontaneously spread to the same sites as do metastatic TNBCs (Figure 3). High expression of nectin-4 was found in two-thirds of PDx, a proportion close to that observed in our clinical series. The results showed a marked efficiency of the anti-nectin-4 ADC with complete and durable eradication of both localised TNBC tumours and metastatic lesions of different sites. These data not only confirmed the results previously reported [17] with another anti-nectin-4 ADC (AGS-22M6E) but also showed additional important results. Our ADC induced complete and durable responses on both primary tumours, metastatic lesions and local relapses, and the degree of response was dependent on the nectin-4 tumour expression level. We surmise that tumour shrinkage is due to toxin delivery because N41mab without drug did not have intrinsic anti-tumour growth activity both in vitro and in vivo. In vivo pre-clinical responses with N41mab-vcMMAE were observed for “nectin-4-high” TN tumours (Q5 > 100), predicting a good response in most TNBC patients (62% of cases). We also found similar efficacy between N41mab conjugated to emtansine (N41mab-DM1) and trastuzumab-DM1 [23] in SUM190 xenografted mice (data not shown). Because both nectin-4 and HER2 are equally expressed at the surface of SUM190 cells, we can postulate that nectin-4-based ADC is at least as efficient as trastuzumab-DM1 in this model.

Efficacy of drugs used for treatment in humans is closely dependent on the toxicity they induce in healthy tissues. This is also true for ADC-based therapy where expression of the target has to be restricted to the malignant cells. Consequently, the restricted expression pattern of nectin-4 in humans would predict a limited toxicity. The ADC tested here did not induce any toxicity in mice even after repeated treatments. However, this cannot predict an absence of toxicity in humans because our N41mab does not cross-react with murine nectin-4. A phase I study addressing this issue was recently completed for examining the safety of AGS-22M6E ADC as monotherapy in patients with malignant solid tumours expressing nectin-4 (Clinical Trials.gov Identifier, NCT01409135). Another ongoing phase I study is assessing the safety and pharmacokinetics of escalating doses of ASG-22CE given as monotherapy in subjects with metastatic urothelial cancer and other malignant solid tumours that express nectin-4 (Clinical Trials.gov Identifier, NCT02091999).

In conclusion, we show that nectin-4 is a new promising therapeutic target in the armamentarium against TNBC. It is a cell surface biomarker frequently expressed in TNBCs, with independent negative prognostic value, and without expression in normal breast tissue. The anti-nectin 4 ADC that we developed (N41mab-vcMMAE) induced complete and durable responses in vitro and in vivo on nectin-4-positive samples including primary tumours, metastatic lesions and local relapses; efficiency was dependent on both the dose and the nectin-4 tumour expression level. Evaluation of this ADC in the clinical setting is warranted. Additionally, because resistance to auristatin-based ADC has been reported [24], we are currently developing in vivo models aiming to evaluate tumour resistance to our ADC.

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Disclosure

The authors have declared no conflicts of interest

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