The Prognostic Value of the Orphan Nuclear Receptor DAX-1 (NROB1) in Node-negative Breast Cancer

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Abstract. Background: DAX-1 inhibits oestrogen receptor (ER) activities and aromatase P450 expression. The prognostic effect of DAX-1 expression in breast cancer was investigated. Patients and Methods: DAX-1, apolipoprotein D (ApoD), mitotic activity index (MAI) and other prognosticators were evaluated, by quantitative immunohistochemistry (IHC) and/or real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis, in 103 invasive node-negative breast carcinomas. Results: With median 122 months follow-up, 24 patients developed distant metastases, of whom, 21 (20%) died. Low DAX-1 expression both by qRT-PCR and IHC was associated with poor survival. Combined strong DAX-1 and ApoD expression identified a subgroup with excellent survival. The most favourable prognostic combination was MAI <3 or (MAI ≥3 combined with DAX-1 and ApoD high expression) versus MAI ≥3 with either low ApoD and/or low DAX-1 expression (p<0.0001, HR=6.1). Conclusion: In operable node-negative breast cancer, strong DAX-1 expression is associated with excellent survival.

Breast cancer is a leading cause of cancer mortality among women in the Western world, second only to lung cancer. Epidemiological features of human breast cancer are consistent with a positive aetiological role for oestrogens (1-3), whose receptors are often overexpressed and function as an oncogene in breast cancer cells. Thus, the oestrogen receptor (ER) status is an important prognostic factor for breast cancer and is often used when deciding endocrine therapy (e.g. tamoxifen (TAM) and aromatase inhibitors). TAM is a selective ER modulator, which inhibits ER signalling in breast cancer cells by enhancing co-repressor recruitment to the promoter region in ERα responsive genes. Aromatase inhibitors block the synthesis of estradiol, the ligand for ERs.

The transcriptional activity of ER requires protein factors that are collectively called co-regulators. These proteins exist in multiple complexes, possess multiple enzymatic activities, and bridge receptors to either chromatin components, the basal transcription machinery, or both. Based on their functional differences, co-regulators are divided into two groups: co-activators and co-repressors. Co-activators are usually recruited to agonist-bound ER and cause ER activation, while co-repressors bind to non-ligated ER and suppress ER activation. However, regulatory mechanisms have been proposed that could play a role in modulation and feedback control of oestrogen signalling (4).

The orphan nuclear receptor DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) is a nuclear receptor protein that in humans is encoded by the NR0B1 gene (nuclear receptor subfamily 0, group B, member 1). The NR0B1 gene is located on the short (p) arm of the X chromosome between positions 21.3 and 21.2. DAX-1 binds to ERα via the N-terminal repeat domain and inhibits the transcriptional activity of ERα in the presence of oestrogen (5). DAX-1 was discovered in 1994 while searching for genes that were related to X-linked adrenal congenital hypoplasia (ACH) associated with hypogonadotropic hypogonadism (6). DAX-1 plays a key role in mammalian sexual differentiation and steroidogenesis. In a normal mouse mammary epithelial cell line, DAX-1 expression was increased in differentiated cells (7). Interestingly, DAX-1 also reduced oestrogen production by inhibiting aromatase expression, thereby down-regulating ER activities (8, 9). DAX-1 also repressed agonist-dependent activities of both the androgen receptor (AR) and progesterone receptor (PR) (10). Strong DAX-1 expression...
caused decreased response to oestrogens in normal HC11 cells (11). An immunohistochemical (IHC) study found strong DAX-1 expression in breast cancer and a positive relationship between the expression of AR, ERα and DAX-1 (12). DAX-1 is therefore closely associated to steroid hormone receptor function in breast cells. However, the prognostic value of DAX-1 in breast cancer is as yet unknown.

Another interesting molecule related to ER regulation is apolipoprotein-D (ApoD), which binds, transports, or chelates lipophylic ligands including TAM. The expression of ApoD is increased in the nipple aspirates from women treated with TAM, possibly through its inhibition of ERα signalling (13). Since ApoD in turn has affinity to TAM, a putative triple inhibitory, clinically relevant relationship between TAM, ERα and ApoD could exist, as has recently been hypothesized (14). The triangular relationship between TAM/ERα/ApoD may explain natural TAM resistance. Thus, when targeting the ERα pathway, the ApoD status of the tumour may be of clinical importance. In agreement with this, in node-positive ERα-positive breast cancer patients older than 70 years, co-expression of ApoD and ERα was associated with a poor prognosis (14). ApoD may predict a decreased effect of TAM in postmenopausal node positive ERα-positive elderly patients (16). However, in women under 70 years or with node-negative breast cancer, ApoD was not prognostic. This may suggest that another factor interfering with ApoD which is present in node-negative carcinomas of women under 71 years is either absent or functions differently in node-positive elderly patients.

The relationship between ApoD and DAX-1 is unknown. In order to investigate the possible biological interactions, whether or not DAX-1 expression in node negative breast carcinomas is correlated with ERα, PR, human epidermal growth factor receptor 2 (HER2), triple negative, mitotic activity index, fibrotic focus and ApoD were evaluated by quantitative IHC and real time reverse-transcription polymerase chain reaction (qRT-PCR) analysis. The additional prognostic value of DAX-1 to these prognosticators was also studied.

 Patients and Methods

Patients and pathology. The study was pre-approved by the Regional Ethics Committee. One hundred and three patients diagnosed with invasive, operable, lymph node-negative (T1,2M0) breast cancer at the Stavanger University Hospital, between January 1, 1993 and December 31, 1997 were analyzed. In 14 tissues, qRT-PCR was inadequate, leaving 89 patients for analysis (there were no differences between the original 103 and the final 89 cases in any of the clinico-pathological features analyzed). The patients were all treated according to the national guidelines of the Norwegian Breast Cancer Group. The tumour size was measured in the fresh specimens following excision and they were then cut into 0.5 cm slices. The axillary fat was macroscopically examined and all detectable lymph nodes were prepared for histology. The median number of identified lymph nodes was 9 (range, 10-36). All the tissues including the invasive front of the tumour were fixed in buffered 4% formaldehyde and embedded in paraffin. Representative samples of the primary tumours were also frozen in liquid nitrogen and stored at –80°C. Four-micrometer histological sections were made and stained with haematoxylin-eosin-safran (H&E). The histological type and grade were assessed by three pathologists (JB, EG, KK) with considerable experience in breast pathology, according to the World Health Organization criteria (17). The grade was carefully assessed according to the Nottingham modification (18, 19). The MAI was assessed as described elsewhere (20). In short, all the unambiguous mitoses were counted in ten consecutive neighbouring fields of vision in the most cellular area (representing a total area of 1.59 mm² at the specimen level) in the invasive front of the periphery of the tumour. The invasive front had to fulfill the following criteria as previously described (21): being the most cellular area; being in the periphery of the tumour and avoiding inflamed or necrotic areas or areas in contact with the epidermis.

Immunohistochemistry. The ER (ERα, ERβ), PR, phosphohistone 3 (PPH3), HER2, ApoD and DAX-1 expression were determined by IHC in whole sections. Antigen retrieval and IHC techniques were based on DAKO technology as described previously (22). In brief, paraffin-embedded sections of 4 μm thickness, adjacent to the H&E sections used for diagnosis, were mounted onto silanized slides (DAKO, Glostrup, Denmark). Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep; Instrumec, Oslo, Norway). The retrieval buffer was 10 mM TRIS/1 mM EDTA (pH 9.0). The sections were heated for 3 min at 110°C followed by 10 min at 95°C and cooled to 20°C. Immunostaining was performed using an autostainer (DAKO), TBS (DAKO) with 0.05% Tween 20 (pH 7.6) was used as the rinse buffer. Endogenous peroxidase activity was blocked by peroxidase blocking reagent (DAKO) for 10 min and the sections were incubated with the antibodies (DAX-1, rabbit polyclonal, Santa Cruz Biotechnology, at 1:300 dilution for 30 min; ApoD clone 36C6, Novocastra (Newcastle upon Tyne, UK) at 1:200 dilution; ERα, clone SP-1, Neomarkers Thermo (Fremont Blvd., Fremont, CA, USA), 1:400 dilution and PR, clone Sp-2, NeoMarkers, 1:400 for 30 min). Rabbit polyclonal anti-phosphohistone H3 (Ser 10) (Lake Placid, NY, USA) was used at a dilution of 1:1500 and the sections were incubated for 60 min at 22°C Dako antibody diluent was used. The immuno-staining was visualized with the DAKO REAL EnVision Detection System, peroxidase/DAB, rabbit/mouse (DAKO) using incubation with EnVision/HRP, rabbit/mouse for 30 min and DAB+ chromogen for 10 min. The sections were counterstained with haematoxylin, dehydrated and mounted.

Scoring protocol for ER, PR and DAX-1. Two independent observers (HZ, EJ) scored the staining intensity in the invasive epithelial cancer cells. If scores differed between the two observers, a consensus score was obtained using a multi-head microscope. The quantitative IHC expression pattern was evaluated by two independent observers, both for the H-score method (23) which is the product of staining intensity (0=none, 1+=weak, 2+=moderate, 3+=strong) and the percentage of cells with a certain staining intensity. The percentage of positive cells was calculated by counting 1000 cells at ×400 magnification. An example of the H-score calculation is: (% cells with 0 staining)×(0) + (% cells with 1+ staining)×(1) + (% cells with 2+ staining)×(2) + (% cells with 3+ staining)×(3)=H-score on a scale 0-300. For ERα and PR, the
percentage of positively stained nuclei was calculated by dividing the number of all positive staining intensities by the total number of counted cells (i.e., 1000). For PPH3, the number of positive nuclei was determined as described before (24). HER2 was assessed according to the manufacturer’s instructions.

Cut-off levels. A count of ≥10% positive cells is commonly used as a threshold for ER and PR positivity, and this was also applied in the IHC part of the present study (other thresholds were investigated, see section statistical analysis, but 10% gave the best prognostic results). For PPH3, the previously assessed threshold of 13 per 10 HPFs was used. ApoD is located in both the cytoplasm and the nucleus, but the combined cytoplasmic and nuclear localization (ApoDCN) versus no such staining has the strongest prognostic value (14) and was therefore used here. Cytoplasmic and nuclear DAX-1 were evaluated separately, using the semi-quantitative H-score.

Real-Time RT-PCR. The total cellular RNA was extracted from the frozen breast cancer tissues with a mirVana miRNA isolation kit (Ambion, Oslo, Norway). RNA samples were evaluated for integrity of 18S and 28S rRNA, and concentration by Bioanalyzer (lab-on-chip). Reverse transcriptions were completed with a TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Carlsbad, CA, USA). Two micrograms of total RNA was transcribed into cDNA in a 100 μl reaction using random hexamers under the thermal conditions recommended by the protocol. Real-time PCR amplification was performed with a Light Cycler 480 (Roche, Zurich, Switzerland), using the DAX-1, ERα, ERβ and ApoD specific primers (Eurofins MWG GmbH, Ebersberg, Germany) shown in Table I. To determine the relative expression level of each target gene, the comparative C_{T} method was used. The C_{T} value of the target gene was normalized by the endogenous reference (ΔC_{T}=C_{T}(target)−C_{T}(GAPDH)) and compared with a calibrator (ΔΔC_{T}=ΔC_{T}(target)−ΔC_{T}(calibrator)). The relative expression of each target gene was calculated via the equation 2^{ΔΔC_{T}}.

Survival end-points. Relapse-free survival (RFS) was defined as the time from the primary operation until confirmation of a systemic relapse (metastasis in any location). Likewise, breast cancer-specific survival (BCSS) was defined as the time from the primary operation until death from breast cancer. The cause of death was provided from hospital records for each patient. “Death from breast cancer” was based on the clinical diagnosis of advanced breast cancer disease supported by radiological and/or cytobhistological information. As the RFS and BCSS end-points gave very similar results, only the BCSS results are discussed.

Statistical analyses. SPSS (SPSS, Chicago, IL, USA) for Windows version 15.0 was used. The optimal expression threshold of the continuous features was determined by CART® data-mining software (Salford sytems, San Diego, CA, USA) and receiver operating curve (ROC) analysis (MedCalc statistical software v. 9.3.7; MedCalc, Mariakerke, Belgium). Correlations between the variables were calculated using the Pearson and kappa tests.
Kaplan–Meier survival curves were constructed and differences between groups were tested by the log-rank test. The relative importance of potential prognostic variables was tested using Cox proportional hazard analysis (forward, backward) and expressed as a hazards ratio (HR) with 95% confidence intervals. Two-tailed \( p \) values <0.05 were considered statistically significant.

Results

Patient characteristics. In the total group of 103 lymph node-negative patients, the median age was 62 (range 34-84 years). With a median follow-up of 122 months (range 14-157 years), 24 patients developed distant metastases of whom 21 (20%) died. The clinicopathological features of the patients are summarized in Table II.

DAX-1 immunoreactivity. DAX-1 was expressed in nearly all the tumours (99.2%), as either cytoplasmic, nuclear or combined nuclear-cytoplasmic (Figure 1). DAX-1 cytoplasmic expression was not prognostic, so only DAX-1 nuclear expression was focused upon. The CART and ROC pointed to H-score 157 and for qRT-PCR determined DAX-1, a relative expression value of 0.02 as the optimal prognostic thresholds which were used in all further analyses. DAX-1 expression measured by IHC and qRT-PCR correlated with each other (\( p<0.05 \)), but as DAX-1 expression measured by IHC was prognostically stronger, this variable was subsequently used.

DAX-1 correlation and survival analysis. DAX-1-IHC and qRT-PCR correlated with ERα (0.02) and PR (0.002), but not with ApoD nor with any of the other features (Table II). Figure 2 shows the Kaplan-Meier survival curves of the patients stratified by IHC DAX-1 expression. Those with strong expression had better survival than those with low or no expression (10-year survival rates: 83 and 52%, absolute survival difference 31%, \( p=0.004 \), hazard ratio=HR 3.1). Strong ApoD expression was also associated with a good prognosis (\( p=0.004 \), HR=4.2). When stratified by ERα, ERβ, PR, and ApoD status, strong DAX-1 expression predicted
better breast cancer specific survival for patients with ER negative \((p=0.04)\), PR-positive \((p=0.009)\) and ApoD-positive tumours \((p=0.03)\). Age added no additional prognostic value to ApoD in these lymph node-negative patients (Cox regression, \(p=0.48\)). MAI was another strong prognostic feature, overshadowing tubule formation and nuclear atypia.

**Multivariate analysis.** With multivariate analysis, strong DAX-1 over-expression combined with strong ApoD expression identified a subgroup of patients with excellent long term survival (91% at 10 years). Figure 3 shows the survival curves for high ApoD expression combined with high DAX-1 expression versus all other combinations \((p=0.004, \text{HR}=4.2)\). However, combined ERα-negativity and low DAX-1 expression was associated with a very poor outcome of 46% at 10 years, \(p=0.04\). The strongest prognostic combination of all features studied was MAI <3 or MAI ≥3 and ApoD high expression and DAX-1 high expression, versus all others (i.e., MAI ≥3 with either low ApoD and/or low DAX-1 expression) (Table III). This resulted in survival rates of 90% and 56% (Figure 4, \(p<0.0001, \text{HR}=6.1\)) respectively.

**Discussion**

Strong DAX-1 expression was correlated with better BCSS, while the patients whose carcinomas had low or no expression had a much less favourable prognosis. Strong DAX-1 overexpression combined with high ApoD expression identified a subgroup of patients with excellent long-term survival. However, combined ERα-negativity or MAI ≥3 with either low DAX1 or ApoD expression identified a subgroup of node-negative breast carcinomas with a very high risk of distant metastatic disease.

The functionality of DAX-1 in breast cancer has not yet been clearly unravelled, even though DAX-1 has been shown to have increased expression in comparison with low...
differentiated mammary epithelial cells (11, 12). DAX-1 is a unique orphan nuclear receptor, which consists of a conservative C-terminal ligand binding domain and an atypical 3.5-repeats N-terminal domain that binds to a number of nuclear receptors. Through bridging co-repressors to the nuclear receptors, DAX-1 modulates nuclear receptor transcriptional activities in a ligand-dependent manner. DAX-1 functions as a negative regulator in the steroidogenesis pathways by inhibiting SF-1, and down-regulating a number of genes such as aromatase (8, 9) and StAR (25). The inhibitory action of DAX-1 on P450 aromatase (8) may explain the favourable prognosis observed in these patients.

Cancer cells in the invasive front of the tumour that express DAX-1 would have a reduced level of mitogenic estrogen ligands available for the ERα. Hence, reduced proliferation and metastatic potential may be the result. Previously, DAX-1 has been shown to function differently in carcinomas from various sites. In two studies, DAX-1 immune-reactivity positively correlated with clinical staging and was associated with a worse prognosis in ovarian cancer and lung cancer (26, 27), contradicting two other studies showing that DAX-1 immune-reactivity inversely correlated with histological grade in endometrial cancer and prostate cancer (28, 29). This could be due to different physiology between different cancer types. In addition, these studies were conducted by IHC only. Therefore, these findings should be regarded as uncertain, unless they are validated by another method such as real-time RT-PCR. In the present study with both IHC and qRT-PCR techniques, strong DAX-1 expression was associated with a good prognosis.

The lack of correlation with ApoD supports the complex regulation of this protein (14). Since ApoD is under control from both the stimulatory AR and inhibitory ER (30), the general steroid receptor inhibition exerted from DAX-1 would not create any net effect on ApoD at the protein level.

Nonetheless, the combination had strong additional prognostic value, further supporting the favourable outcome mentioned above in relation to DAX-1.

Recently, another splice variant of DAX-1, called DAX-1A, has been discovered. The currently available antibodies actually recognize both forms of DAX-1. In addition, the DAX-1 antibody used in this study might not have been totally specific. However, the results were validated by qRT-PCR which correlated with the IHC results.

The relationship between DAX-1 and the P450 aromatase should be further explored, since DAX-1 may be a natural intrinsic aromatase inhibitor possibly defining a subgroup of node negative patients not in need of adjuvant treatment with aromatase inhibitors. MAI was another strong prognostic feature, overshadowing tubule formation and nuclear atypia. The same results were obtained in another, independent, study (31). Interestingly, combined low proliferation (i.e. MAI <3), or high proliferation combined with strong ApoD and DAX-1 expression was associated with excellent survival of >90% at 10 years.

In conclusion in node-negative invasive breast carcinoma, strong DAX-1 expression, especially when combined with strong ApoD expression is associated with excellent survival. Combined ER-negativity or MAI ≥3, and either low DAX1 or ApoD expression identifies a subgroup of node-negative breast carcinomas with a very high risk of distant metastatic disease.

**Acknowledgements**

We thank Stavanger Health Research for financial support. This study was supported in part by grant 09-12 of the SBDM. We also thank our colleagues at the Molecular Quantitative Pathology unit of the Department of Pathology for the technical support, especially Bianca van Diermen. Special thanks to Susan Allen for correction of the English text.

**References**


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**Table III. Correlations between DAX-1 (as H-Score <157 versus ≥157) and clinicopathological variables.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>DAX-1 p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (&lt;55, 55-70, &gt;70 years)</td>
<td>0.14</td>
</tr>
<tr>
<td>Tumour diameter (&lt;2, ≥2 cm)</td>
<td>0.20</td>
</tr>
<tr>
<td>MAI (&lt;3, 3-9, &gt;9)</td>
<td>0.11</td>
</tr>
<tr>
<td>MAI (&lt;3, ≥3)</td>
<td>0.07</td>
</tr>
<tr>
<td>ERα (Neg, Pos)</td>
<td>0.02</td>
</tr>
<tr>
<td>ERβ (Neg, Pos)</td>
<td>0.63</td>
</tr>
<tr>
<td>PR (Neg, Pos)</td>
<td>0.002</td>
</tr>
<tr>
<td>HER2 (0-2 versus 3)</td>
<td>0.16</td>
</tr>
<tr>
<td>Fibrotic focus</td>
<td>0.59</td>
</tr>
<tr>
<td>ApoD (Neg, Pos)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

MAI: mitotic activity index.

**Table IV. Multivariate analysis, combining MAI and DAX 1-ApoD.**

<table>
<thead>
<tr>
<th>Step 1 variable</th>
<th>Beta</th>
<th>Standard Error</th>
<th>P-value</th>
<th>Hazard Ratio</th>
<th>95% CI for HR Lower</th>
<th>95% CI for HR Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>MAI-3</td>
<td>2.0</td>
<td>0.74</td>
<td>0.006</td>
<td>7.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Step 2</td>
<td>MAI-3</td>
<td>2.0</td>
<td>0.74</td>
<td>0.007</td>
<td>7.4</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>ApoD-DAX1</td>
<td>1.4</td>
<td>0.55</td>
<td>0.012</td>
<td>4.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Nonetheless, the combination had strong additional prognostic value, further supporting the favourable outcome mentioned above in relation to DAX-1.