Abstract. Background: Cul-4, a member of the Caenorhabditis elegans “cullin” ubiquitin-ligase gene family, plays a critical role in regulation of DNA-replication in this nematode. It has been suggested that cul-4 might have an important role in the development and progression of human cancer, but no data on this subject exist. The aim of this study was to investigate the expression and prognostic relevance of CUL-4 protein in lymph node-negative breast cancer, one of the most common malignancies worldwide. Materials and Methods: CUL-4 protein expression was determined with immunohistochemistry in 167 specimens of human node-negative invasive breast cancer with long term follow-up. Results were correlated with overall and disease-free survival of patients. Results: Strong expression of CUL-4 protein was observed in 32 cases (19.2%), moderate expression in 59 (35.3%), weak expression in 64 (38.3%), and in 12 tumors (7.2%) no expression of CUL4 was observed. Patients with strong expression of CUL4 had a significantly shorter overall and disease-free survival (p=0.04 and p=0.029, respectively; Cox regression) compared to all other cases. Conclusion: Our data provide evidence for the first time that CUL-4 could play an important role in the development and progression of human cancer.

In order to maintain genome stability, DNA replication is strictly regulated to occur only once per cell cycle. Cul-4, a member of the Caenorhabditis (C.) elegans “cullin” ubiquitin-ligase gene family (1), a family of highly conserved genes, seems to play an important role in this regulation (2). CUL-4 has been shown to restrict DNA-replication licensing in C. elegans (3). Although it has been suggested that the human homologue of cul-4 may play a critical role in cancer progression (4, 5), to our knowledge no published data on its clinical relevance exist so far. The aim of this study was to evaluate the prognostic relevance of CUL-4 protein expression in lymph node-negative human breast cancer, one of the most common human cancers worldwide.

Materials and Methods

Patients. For immunohistochemistry, 167 consecutive formalin-fixed, paraffin-embedded samples of lymph node-negative invasive breast cancer, UICC stages 1-4, were retrieved from our files. All tumors were re-graded according to Elston (6). For estrogen receptor density measurement snap-frozen tumor samples from the same patients were used. All patients had at least 10 lymph nodes isolated from the axillary fatty tissue.

The vast majority of patients were treated within prospective clinical trials and were therefore followed-up accordingly (7).

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded specimens fixed in 4% buffered formalin. Histological sections, 4 µm in thickness, were deparaffined in xylene. Endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 min. For immuno- histochemistry, a DAKO autostainer (DAKO, Glostrup, Denmark) was used. Specimens were incubated for 1 hour at room temperature with a polyclonal rabbit antibody against human CUL-4 (sc-10782, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:15. This antibody was raised against amino acids 536-597 mapping near the C-terminus of CUL-4 of human origin and detects GST-tagged human recombinant CUL-4 by Western blotting (not shown.)

Bound antibodies were visualized using streptavidin-biotin-peroxidase complex (ChemMate kit, DAKO), using diaminobenzidine as chromogene. As a positive control, a specimen of breast cancer with known overexpression of CUL-4 was used. For a negative control, the primary antibody was replaced by non-immune, normal rabbit serum.

The expression of CUL-4 was relatively uniform throughout each individual tumor specimen and was graded as “negative”, “weak” expression, “moderate” expression and “strong” expression (Figure 1). Since weak to moderate expression of CUL-4 was found regularly in the epithelium of normal breast parenchyma (Figure 1...
A), it served as an internal positive control. A tumor specimen was only considered as “strongly” expressing CUL-4 if the vast majority of tumor cells expressed this protein considerably stronger than normal breast tissue.

Analysis of immunohistochemistry was carried out by two independent observers. If differences among observers occurred, these slides were reinvestigated by both investigators using a multiheaded microscope.

Estrogen receptor density was determined using the dextran charcoal method from snap-frozen tumor samples as described elsewhere (8). For definition of estrogen-receptor positivity, a cut-off value of >10 fmol/l was used (9).

Statistics. The Chi-square test, Mann-Whitney test and Spearman’s coefficient of correlation were used as appropriate. Overall survival (OS) was defined from the day of surgery until the death of the patient. Death from a cause other than breast cancer, or survival until the end of the observation period were considered as censored observations. Disease-free survival (DFS) was defined from the day of primary surgery to the first evidence of local or distant recurrent disease. Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier (10). The Cox proportional-hazards model was used for multivariate analysis. CUL-4 expression (absent-moderate vs. strong expression), patients age at time of diagnosis, histological grading, tumor stage (UICC), estrogen receptor status and menopausal status were entered into Cox-regression. For all tests, a two tailed p-value of ≤0.05 was considered as significant.

Results

The mean age of cancer patients at time of surgery was 60.4±13.7 years. Forty-six patients (27.5%) were premenopausal, 121 (72.5%) postmenopausal. One-hundred and forty-nine cases were classified as invasive ductal carcinoma and 18 (10.8%) as lobulary carcinomas. Eighty-four tumors (50.3%) were classified as UICC stage 1, 67 (40.1%) as stage 2, 2 (1.2%) as stage 3 and 14 (8.4%) as stage 4.

Mean estrogen receptor density was 132.3±264.5 fmol/liter, 114 patients (69.1%) were considered as estrogen-receptor positive. As surgical treatment, breast conservation (usually wide excision) was performed in 79 patients (47.3%) and mastectomy in 64 (38.3%), while in 24 patients no data on exact type of surgery were available. After breast
conservation, the majority of patients were treated with adjuvant radiotherapy, except a small subgroup of patients with minimal risk. Twenty-one patients (12.6%) received no adjuvant therapy. In 89 patients, (54.9%) Tamoxifen was administered for 5 years at a dose of 20 mg/day. A combined adjuvant chemotherapy (6 x CMF intravenously for 6 cycles, days 1 and 8, recycled on day 28, at doses of: cyclophosphamide 600 mg/m², methotrexate 40 mg/m², and fluorouracil 600 mg/m²) was administered in 36 patients (21.6%). Two patients (1.2%) received CMF-chemotherapy plus tamoxifen. Seven patients (4.2%) received goserelin for 3 years (3.6 mg s.c. every 28 days) plus tamoxifen (29 mg/day) for 5 years. Four patients (2.4%) received tamoxifen (20 mg/day) plus aminoglutethimide (500 mg/day) for 2 years. In 4 patients, no information on adjuvant therapy was available.

CUL-4 staining signals were seen mostly in the cytoplasm of cells, but sometimes in addition positive nuclear signals were observed. Strong expression of CUL-4 protein was observed in 32 cases (19.2%) (Figure 1B). Moderate expression was seen in 59 cases (35.3%) (Figure 1C), while weak expression was found in 64 specimens (38.3%) (Figure 1D), but in 12 tumors (7.2%) no expression of CUL-4 was observed.

No association of CUL-4 expression with histological grading, tumor type, tumor stage, estrogen receptor density or patients’ age was observed (p > 0.05).

The median follow-up time of patients was 100 months (range: 1-187 months). During the observation period, 63 patients (37.7%) developed recurrent disease and 49 patients (29.3%) died from their breast cancer.

When comparing the OS of patients with strong expression of CUL-4 to all others, a significantly shorter OS was found using univariate analysis (p=0.0054, log-rank test, Figure 2A). The 10-year OS rate was 68.6% in patients without strong CUL-4 expression, while it was 46% in those with strong CUL-4 expression.

Upon univariate analysis of DFS, CUL-4 was of prognostic relevance (p=0.006, log-rank test) (Figure 2B). The 10-year DFS rate was 59.3% in patients without CUL-4 overexpression while it was only 42.6% in those with CUL-4 overexpression.

In multivariate analysis, strong CUL-4 expression was an independent prognostic factor for OS and DFS (Table I).

Discussion

Duplication of the genome and equal segregation of replicated DNA to daughter cells are central objectives in cell cycle regulation (11). Any disturbance of genome replication hampers stability of the genome and results in cell death or diseases like cancer.

It has been shown that inactivation of a single gene, *cul-4*, leads to massive DNA-re-replication in *C. elegans* (3). Therefore, it appears reasonable that this gene regulates multiple aspects of DNA-replication licensing and plays a key role in the whole cell-cycle.

Due to its central role in preventing genomic instability and the fact that this genomic instability is a key factor in the development of cancer (12), CUL-4 might also play an important role in the development and progression of human malignancies. In mammalians, two orthologs of *cul-4* exist: while *cul4A* seems to promote nucleotide excision repair, the function of *cul4B* is still unclear (11), although due to the highly conservative nature of “cullin” genes, a very similar or even identical function might be anticipated.

Nevertheless, some data on the expression of CUL-4 in human cancers exist: Chen et al. showed that *cul4A* gene was amplified and mRNA overexpressed in 3 out of 14 human breast cancer cell lines, and mRNA was overexpressed in 8
Table I. Analysis of survival of 167 patients with node-negative breast cancer.

<table>
<thead>
<tr>
<th></th>
<th>Significance univariate</th>
<th>Significance multivariate</th>
<th>95% confidence interval</th>
<th>Relative risk</th>
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<tr>
<td>Overall survival</td>
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<tr>
<td>CUL-4 (absent-moderate)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>vs. strong</td>
<td>0.0054</td>
<td>0.040</td>
<td>1.03-4.27</td>
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<td>0.013</td>
<td>1.13-2.86</td>
<td>1.80</td>
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<td>Patients' age</td>
<td>&lt;0.0010</td>
<td>&lt;0.001</td>
<td>1.03-1.1</td>
<td>1.07</td>
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<tr>
<td>Tumor stage (UICC)</td>
<td>0.6573</td>
<td>0.247</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Estrogen receptor positive (yes vs. no)</td>
<td>0.1894</td>
<td>0.291</td>
<td>–</td>
<td>–</td>
</tr>
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<td>Menopausal status</td>
<td>0.0054</td>
<td>0.558</td>
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<td>–</td>
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<tr>
<td>Disease-free survival</td>
<td></td>
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<tr>
<td>CUL-4 (absent-moderate)</td>
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<tr>
<td>vs. strong</td>
<td>0.0060</td>
<td>0.029</td>
<td>1.07-3.45</td>
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<td>Histological grading</td>
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<td>Patients' age</td>
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<td>0.087</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Menopausal status</td>
<td>0.0720</td>
<td>0.312</td>
<td>–</td>
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</table>

More work is required to analyze the functional role of CUL-4 in different types of human cancer. When antibodies able to discriminate between CUL-4A and CUL-4B proteins become available, it would be of great interest to determine if both proteins are overexpressed simultaneously in human cancer specimens. It will also be of considerable interest to find out more about proteins interacting with CUL-4. Not only CUL-4 itself, but also these putative proteins might represent targets for novel, selective therapeutic strategies.

References


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