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# Original article

## Estrogen dependent expression of the receptor tyrosine kinase axl in normal and malignant human breast

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**Summary**

*Background:* Axl, a member of a family of receptor tyrosine kinases characterized by an extracellular domain resembling cell adhesion molecules and an intracellular conserved tyrosine kinase domain has been reported to induce cell proliferation and transformation. In mice, axl is expressed in the normal mammary gland and over-expressed in aggressive mammary tumors.

*Patients and methods:* We have investigated the expression of axl immunohistochemically in 23 normal human breast samples and in 111 consecutive breast carcinomas. Expression of axl was correlated with tumour characteristics (lymph node involvement, stage, grade) and immunohistochemical expression of ER, PR, Ki-67 and c-erbB-2.

*Results:* In normal tissue, axl localizes to the membrane of breast epithelial cells. Axl protein shows membrane associated staining in high correlation ($P = 0.004$) with the expression of the estrogen receptor (ER). Axl expression was found in a subset of breast carcinomas and was also correlated with high significance ($P < 0.0001$) with the presence of ER.

*Conclusion:* Our results suggest that axl may serve as a mediator of estrogen stimulation preventing the completion of the breast epithelial life cycle and that estrogen induced axl expression may give a survival signal to cancerous cells, preventing them from dying through apoptosis.

**Key words**: apoptosis, breast cancer, estrogen receptors, immunohistochemistry

### Introduction

Breast cancer is one of the most common malignancies in women in the Western world with an approximate probability of one in ten women developing the disease. The vast majority of breast cancers are sporadic and their etiology is largely unknown. Exposure to estrogen has been identified as one of the major risk factors. Early menarche, regular ovulatory cycles and late menopause are associated with increased risk for development of the disease. Prolonged exposure to the periodic developmental processes occurring during the estrous cycle without subsequent differentiation, like in nulliparity of late first full-term pregnancy also increases risk [1]. Two additional observations support the influence of estrogen on growth control of the breast epithelium. First, the risk of having breast cancer diagnosed is increased in women using estrogen during hormone replacement therapy and this risk increases with the duration of use [2]. Second, anti-estrogen treatment of women at high risk for developing breast cancer decreases the incidence of the disease [3]. A better understanding of the mechanisms controlling hormone-induced growth of the breast epithelium is desperately needed for the development of new prognostic and therapeutic tools allowing an improved clinical management of the disease.

The growth of the breast is unique compared to most other organs beginning its development only through the hormonal stimulation at puberty. Moreover, end-differentiation of the breast epithelium is reached only upon full term pregnancy. In the absence of pregnancy, hormonal changes throughout the menstrual cycle induce the breast epithelium to limited proliferation, differentiation and cell death by apoptosis [4]. Any escape from this tightly regulated equilibrium of growth and regression may eventually result in malignant transformation. Indeed, it has been shown that rat mammary epithelium (the breast epithelium) is especially prone to neoplastic development during the follicular phase of the cycle [5], when estrogen stimulates epithelial proliferation. As shown for the human breast, the proliferating epithelial cells themselves do not posses estrogen receptors indicating that the proliferative effect of estrogen is indirect and requires intercellular mediators [6]. Little, however, is known about the regulatory mechanisms in the breast during the menstrual cycle.

It is now well established that protein tyrosine kinases (PTKs) play an important role in the regulation of cellular proliferation and differentiation and in the genesis of many neoplasias. PTKs are generally characterized as transmembrane receptors or intracellular non-receptor type signal transducers [7]. Ligand binding by the extracellular domain of the membrane spanning receptor PTKs activates the intracellular tyrosine kinase domain

and initiates specific signal transduction pathways [8]. The hormone-responsive regulators of the breast epithelium are largely unknown; however, the established role of PTKs in the control of cell proliferation makes them prime candidates for an involvement in mediating hormone induced growth signals and thus also in the development of mammary gland neoplasias. Indeed, an indirect influence of estrogen on PTK has been described for the epidermal growth factor receptor, the Eph receptor family member EphB4, and for a non-receptor tyrosine kinase, iyk [9–12].

In a survey of PTKs expressed in the mouse mammary gland throughout the estrus cycle, we have analyzed the RNA expression of the receptor tyrosine kinase *ark*, the murine prototype of *axl*, during mammary gland development, the estrous cycle and carcinogenesis [13]. *Axl* is a member of a small family of receptors with an extracellular domain resembling cell adhesion molecules and a conserved tyrosine kinase domain [14]. *Axl* has been reported to be implicated in homophilic cell aggregation [15] and as a receptor for the Gas6 ligand [16]. Furthermore, it has been shown that *axl* activation induces cell proliferation and transformation [16]. In mice, axl was differentially expressed in the normal mammary gland during the estrous cycle with a maximum at met-estrous. A strong over-expression could be shown in the aggressively growing Wap-*ras*-induced mammary tumours [13]. These observations prompted us to investigate axl expression in the normal and malignant human breast. Here, we report that axl expression in normal breast tissue is restricted to the epithelial component of the gland, where the axl protein shows membrane associated staining in high correlation with the expression of the ER.

## Patients and methods

### Patient material

The material used in this study was taken from patients treated between August 1997 and May 1999. Malignant breast samples were obtained from primary breast lesions. The stages of the invasive carcinomas were classified as based on the TNM classification. For normal human breast samples, tissue specimens were obtained from reduction mammoplasties. The hormonal state of the patients were determined by questioning. Patients receiving hormonal substitution or with unclear cyclic states were not included in this study. This study was approved by the ethical commission of the University of Berne.

### Immunohistochemistry

Breast tissue specimens were fixed in 4% formaldehyde and paraffin-embedded; 4 µm sections from the same sample were de-waxed, rehydrated and washed 3 times for 5 min at room temperature in TBS (25 mM Tris-HCl, pH 7.5; 140 mM NaCl). Sections were boiled in a microwave oven in 10 mM citrate buffer, followed by washing in TBS (3 times for 5 min), and endogenous peroxidase was blocked by incubation in 0.3% $H_2O_2$ in TBS for 10 min at room temperature. Incubation with the primary antibodies was done overnight at 4 °C. Antibodies were diluted in either 5% TNA (50 mM Tris-HCl, 140 mM NaCl, 0.5% Na caseinate, 15 mM $NaN_3$) (anti-axl, 1 : 100; anti-c-erbB-2, 1 : 50) or TBS (anti-ER, 1 : 25; anti-progesterone receptor (PR), 1 : 25; anti-Ki-67, 1 : 100). All antibodies were obtained commercially: anti-axl (Santa Cruz Biotechnology, Santa Cruz, California); anti-ER, anti-PR, anti-Ki-67, anti-c-erbB-2 (DAKO A/S, Glostrup, Denmark). After three washes in TBS, sections were incubated with biotinylated swine anti-rabbit or rabbit anti-mouse antibodies (DAKO) for 1 hr at room temperature. After three washes in TBS, sections were incubated with avidin and biotin-horseradish peroxidase complex or alkaline phosphatase complex. Peroxidase activity was localized by incubation in substrate solution [3,3'-diaminobenzidine tablets (Sigma, Buchs, Switzerland) dissolved in 100 mM imidazole, 100 mM NaCl, 20 mM citric acid (pH 7.0) containing 0.005% $H_2O_2$] for 8 to 10 min at room temperature. Phosphatase activity was localized by incubation in naphtol and fuchsine substrate solution for 20 min at room temperature. Sections were counterstained with haemalaun for 30 seconds and mounted in Aquatex (Merck, Darmstadt, Germany). Control sections included omission of the primary antibody.

### Statistical analysis

The number of immunoreactive cells was semi-quantitatively estimated by two independent investigators (G.B and H.J.A) who were blinded with regard to the results of other markers. + corresponded to < 10% positive cells, ++ to 10%–50% positive cells and +++ to > 50% positive cells. For every sample, at least 100, usually > 1000 cells were analyzed. Samples with > 10% positive cells were deemed positive. SAS statistical software was used for statistical analysis. The statistical significance of differences between individual groups was analyzed using Fisher's exact test. The relation between the dependent variable axl and the other independent variables was examined using the multiple logistic regression model for polytomous data with ordinal scale [17]. The full model was reduced by a backward elimination procedure to get to the final model. Spearman's rank correlation coefficients were used to describe pairwise relations between variables c-erbB-2, Ki-67, PR and ER. Analyses of survival were performed using the Kaplan–Meier method. Survival distributions were compared with the log-rank test. $P < 0.05$ was considered statistically significant.

## Results

### *Expression in the normal human breast*

We have investigated axl expression in normal human breast specimens at the proliferating follicular and the differentiating luteal phases of the menstrual cycle, as well as at the resting phase after menopause. Tissue specimens were obtained from 23 normal human breast samples including 6 post-menopausal and 17 pre-menopausal patients. Twelve patients were in the follicular phase of the menstrual cycle and five in the luteal phase. The mean age of these 23 patients was 40.3 ± 12.5 years (range 18–70). All pre-menopausal patients had a cycle time between 28 and 31 days.

These experiments revealed that axl is exclusively expressed in the breast epithelium. No axl protein was detected in myo-epithelial cells, fibroblasts surrounding the breast parenchyma or in fibro-adipose tissue. In the epithelial cells, membrane associated staining was seen (Figure 1a). This staining was not present when the first antibody was omitted. During the follicular phase of the menstrual cycle, a higher number of epithelial cells was axl positive compared to the luteal phase. As hormonal status strongly influences the physiology of the breast,

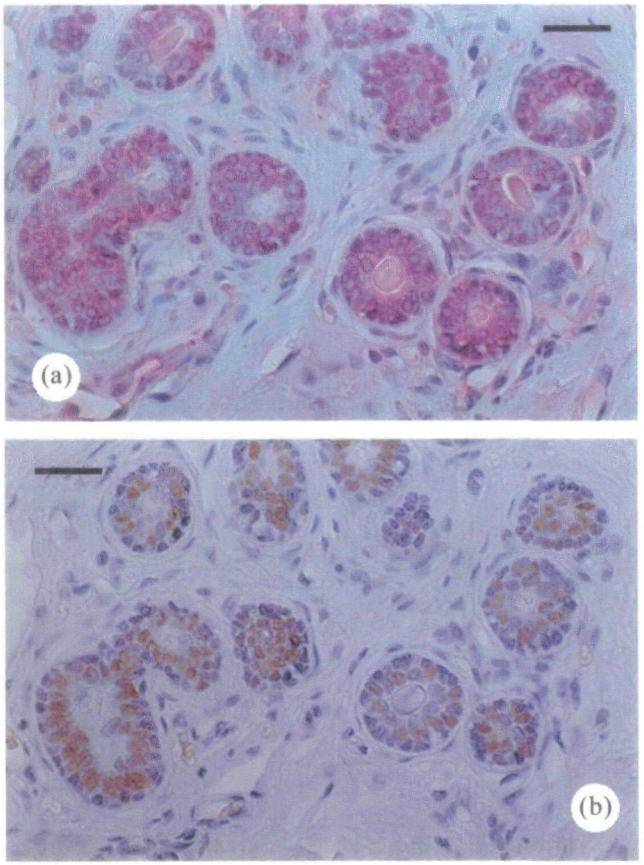


*Figure 1.* Localization of axl protein and estrogen receptor (ER) in normal breast tissue. Sections were reacted with anti-axl (a) or anti-ER antibodies (b), visualized with alkaline phosphatase (a) or horse radish peroxidase (b). Scale bars = 50 μ.

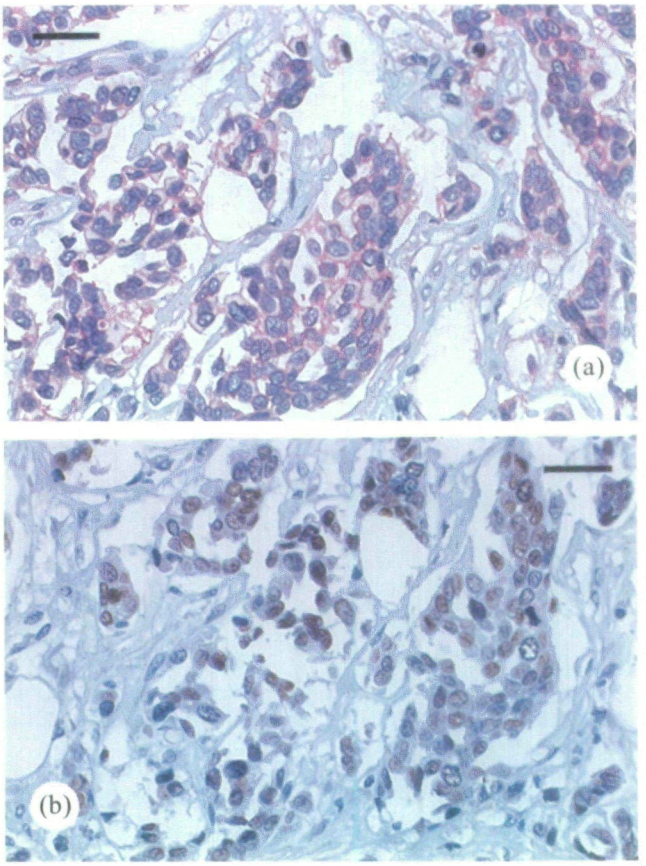


*Figure 2.* Axl and estrogen receptor (ER) expression in an invasive ductal carcinoma. Sections were reacted with anti-axl (a) or anti-ER antibodies (b), visualized with alkaline phosphatase (a) or horse radish peroxidase (b). Scale bars = 50 μ.

we have analyzed the expression of ER (Figure 1b) which was found more often during the follicular than during the luteal phase of the cycle ($P = 0.046$). Strikingly, we have found a highly significant association ($P = 0.004$) of axl positivity with the ER status in the normal human breast epithelial cells.

*Expression of axl in malignant disease of the human breast*

The analysis of the normal human breast has indicated a strong correlation between axl and ER expression. Although being far from unerring, the status of the ERs is the most relevant factor predicting treatment response to endocrine therapy in breast cancer [18]. We have therefore investigated axl expression as a potential mediator of estrogen stimuli in malignant disease of the breast. Malignant breast samples included 111 consecutive invasive carcinomas (87 ductal, 20 lobular, 3 mucinous and 1 medullary carcinomas) and 3 ductal *in situ* carcinomas. The clinical stages of the invasive carcinomas were classified as follows: 55 as pT1, 44 as pT2, 10 as pT3 and 2 as pT4. Eight (7%) of our patients tumours were classified as grade 1, 78 (68.4%) as grade 2 and 28 (24.6%) as grade 3. Lymph-node dissection was performed on 99 patients with invasive carcinoma, 40 of whom were positive for metastases. Mean age at diagnosis for the carcinoma group was 59.4 ± 13.5 years (range 31–81) and 81 from 114 patients (71%) were postmenopausal. The median follow-up of the patients without recurrence is 25 ± 8 months (11–42 months).

Immunohistochemical staining revealed membrane localization of the axl protein in neoplastic cells of a subset of tumours analyzed. Like in the normal breast, we observed a high correlation between axl and ER expression (Figures 2a and 2b). These results, including the results of immunohistochemical analysis of PR expression, are summarized in Table 1. Interestingly, axl expression was only found in ER positive tumours, however, not all ER positive tumours expressed the axl protein.

*Relation between axl expression and prognostic markers*

In a multiple logistic regression (Table 2), we could find a highly significant relation between axl positivity and ER ($P < 0.0001$). A significant relation was also found between axl and the tumour stage ($P = 0.006$) and the immunohistochemical expression of the proliferation marker Ki-67 ($P = 0.022$). There was no relation with other prognostic factors, such as grade of tumour ($P = 0.711$), lymph node involvement ($P = 0.740$), and PR expression ($P = 0.414$) or with the clinical outcome such as shorter disease-free survival ($P = 0.94$). As expected,

*Table 1.* Epithelial expression of axl, estrogen receptors (ER) and progesterone receptors (PR) in human breast carcinomas.

| | < 10% | 11%–50% | > 50% | *n* |
|---|---|---|---|---|
| Axl | 37 | 38 | 39 | 114 |
| ER | 27 | 16 | 71 | 114 |
| PR | 44 | 28 | 42 | 114 |

*Table 2.* Relation between membrane accumulation of axl and prognostic factors in breast cancer ($n$ = 113).

| | In[a] | Chi-square[b] | *P*-value |
|---|---|---|---|
| Summary of backward elimination | | | |
| Grade of tumour (G) | 6 | 0.68 | 0.711 |
| Menopause | 5 | 0.60 | 0.438 |
| PR | 4 | 0.66 | 0.414 |
| Age | 3 | 1.45 | 0.227 |
| Final model | | | |
| Tumour stage | | 14.12 | 0.006 |
| Ki-67 | | 5.23 | 0.022 |
| ER | | 29.84 | < 0.0001 |

[a] Number of independent variables remaining in the model after the examined variable is removed from the model. Value of the statistic test to calculate the *P*-value.

The relevant parameters for the antibody staining of axl (+/++/+++) were examined using the multiple logistic regression model for polytomous data with ordinal scale [17]. The full model (grade of tumour, 1/2/3; menopause, yes/no; PR, +/++/+++; age, years; tumour stage, pTis to pT4; Ki-67, +/++/+++; ER, +/++/+++) was reduced by a backward elimination to the final model. The final model provides a set of variables that have a high influence on the dependent variable axl.

there was a strong association of ER status with PR status ($P$ = 0.0001) and a significant inverse relationship between ER status and Ki-67 ($P$ = 0.008) or c-erbB-2 ($P$ = 0.002) (Table 3).

*Table 3* Correlation between antibody staining of estrogen receptors (ER), progesterone receptors (PR) and new prognostic factors (Spearman's rank test).

| | Correlation-coefficient (two-tailed *P*-value) | | | |
|---|---|---|---|---|
| | ER | PR | Ki-67 | c-erbB-2 |
| ER ($n$ = 114) | 1.000 (–) | | | |
| PR ($n$ = 114) | 0 435 (0 0001) | 1.000 (–) | | |
| Ki-67 ($n$ = 113) | –0.246 (0 008) | –0.233 (0.013) | 1.000 (–) | |
| c-erbB-2 ($n$ = 105) | –0.302 (0 002) | –0 166 (0.091) | 0.215 (0 028) | 1 000 (–) |

## Discussion

The immunohistochemical investigation of normal breast tissue has revealed that the axl protein, the human homologue of mouse ark, localizes in the membrane of the epithelial cells. Expression of proteins of the *axl* family is predominantly localized to the central nervous system, but they are also notably expressed in many non-neural tissues such as kidney, spleen, ovary and testis [14]. In tissue culture, axl is expressed in different cell lines of epithelial cell origin [19]. In the mouse mammary gland, the expression of a family member, sky, is regulated during pregnancy, lactation and involution, suggesting its involvement in normal mammary gland development [20]. Furthermore, axl shows a differential expression during the estrous cycle, supporting the postulated role in normal mammary gland physiology [13]. In mouse mammary tumours, a possible involvement in invasive carcinogenesis is suggested by the strong over-expression of axl only in the aggressively growing Wap-ras induced mammary tumours and by the expression in several human breast cancer cell lines [13]. Moreover, strong expression in mouse mammary tumours and overexpression in a tumourigenic human breast cell line has been described for the other family member, sky [20]. In our study, axl was localized in the membrane of the human breast cancer cells and the number of cells expressing axl was found to be higher in cancerous tissue than in the normal breast ($P$ < 0.001). Overexpression of axl has also been observed in a metastatic sample of a colon cancer [21].

We have found a significant association ($P$ = 0.004) of axl positivity with the ER status in normal human epithelial breast cells and in breast cancer cells. In the mouse mammary gland, axl was already detected in immature females but showed a maximal expression in mature females during the estrous and the met-estrous phase, characterized by maximal estrogen secretion. During an estrous, which is characterized by alveoli with a flattened epithelium, regional cell death and minimal estrogen secretion, only low axl expression was detected [13]. We have also observed a higher expression of ER and axl during the estrogen dominated follicular phase of the cycle in the human breast, but this difference was statistically not significant. This result is not surprising since the developmental processes occurring in the breast during the menstrual cycle involve only a subset of lobular structures [22]. Furthermore, it has been shown that the percentage of cells which express ER in the normal human breast not only varies throughout the menstrual cycle but also greatly varies during the different phases of the cycle [23]. Our results suggest the importance of estrogens for the presence of axl proteins. Although the effect of progesterone on the expression of axl expression remains to be investigated, the onset of its expression in immature mouse females, when progesterone is not yet active [24], the high expression in the follicular phase and the absence of association with PR in breast cancers support the notion that estrogen represents the main inducer of axl protein.

In a recent study, the influence of estrogens on another receptor PTK, EphB4 was demonstrated [10]. An indirect effect of the activated estrogen receptor on the translation and/or stabilization of the EphB4 receptor and its ligand has been suggested. Estrogens can also promote the autocrine expression of insulin-like growth factor-II (IGF-II) [25] and epidermal growth factor receptor (EGFR) [26] and interact with both c-erbB2 protein and the nuclear receptor by a similar molecular

mechanism [27]. Thus, the interactions between steroid hormone receptors and receptor PTKs may be of importance for modulation and/or targeting of hormone induced signals.

Axl was first isolated as an oncogene with a weak oncogenic activity and transformation could only be observed in cells overexpressing axl protein [19]. Furthermore, very high doses of Gas6, the ligand of axl have been shown to induce cell proliferation [14, 28]. This weak mitogenic activity is supported in our study by the association ($P = 0.022$) between axl and the proliferation index determined by the expression of Ki-67 in breast cancer. Interestingly, constitutive expression of Gas6 in NIH3T3 prevents cell from undergoing apoptosis without inducing cell proliferation [29]. Cell death or apoptosis in serum deprived growth arrested NIH3T3 cells is also prevented by addition of Gas6 and this effect is independent of its mitogenic activity [28]. Furthermore, embryonic fibroblasts from axl knock-out mice are highly susceptible to apoptosis induced by serum deprivation [29]. As the antiapoptotic effect of Gas6 is reached at concentrations ten times lower than those needed to stimulate mitogenesis, it is possible that the true role of axl signaling consists in promoting cell survival [29]. Our results indicate that ER probably induce axl expression and this interaction could play an important role in the proliferation, differentiation and apoptotic cell death in human breast epithelium during the menstrual cycle. Conceivably, increased local estrogen activity inducing axl expression may be a trigger of mammary carcinogenesis by giving a survival signal to cancerous cells, preventing them from apoptotic cell death and thereby from completion of their life cycle. This promotion of cell survival is realized under conditions which do not involve high cell proliferation.

*Bcl-2* is one of the few genes thought to be involved in the control of cell survival and cell death during the estrous cycle [4]. Like axl, expression of the *bcl-2* gene has been shown to counteract apoptosis and to prolong cell survival without affecting cell proliferation [30].

Consistent with this potential to inhibit apoptosis, overexpression of *bcl-2* in Wap–*bcl-2* mice was able to complement an increased expression of myc and promote mammary tumorigenesis. Anti-estrogen treatment is associated with increased apoptosis in hormone-dependent breast cancer cell lines growing *in vitro* or *in vivo* [31, 32]. The anti-estrogen tamoxifen possesses the ability to induce apoptosis in the estrogen dependent breast cancer cell line MCF-7 probably through down-regulation of *bcl-2* expression [33]. It remains to be elucidated, if the ER induced axl signaling is involved in the control of anti-apoptotic proteins such as *bcl-2*.

In summary, our results revealed a strict dependence of axl expression on the presence of ER. The fact that not all ER positive cells were also positive for the axl protein suggests that this cell population has distinct properties than the axl negative ones. This fact also raises the possibility that axl expression influences the response of cancer cells to anti-estrogen treatment. It remains to be elucidated if axl expression may represent a novel marker predicting the response of ER positive tumours to hormonal therapy. These studies, however, require further retrospective investigations including a larger number of patients with a longer follow-up than it was used in this pilot study.

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