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PRENEOPLASTIC MAMMARY TUMOR MARKERS: CRIPTO AND AMPHIREGULIN ARE OVEREXPRESSED IN HYPERPLASTIC STAGES OF TUMOR PROGRESSION IN TRANSGENIC MICE

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Amphiregulin (Ar) and Cripto (Cr) are autocrine growth factors for mammary cells and both have been observed to exhibit high expression in human mammary tumors, in contrast with adjacent tissues. To investigate whether Ar and Cr play roles in the progression of mammary cell proliferation to unregulated growth and tumor formation, the levels of expression were examined in transgenic mice (TGM) that over-express several different oncogenes: MMTV-Polyoma virus middle T antigen (MMTV-PyMT), MMTV-c-ErbB2 (c-neu, HER2) and MT-hTGFα. These transgenic mice all produce mammary tumors but with different rates of progression. The levels of Ar were induced up to 10-fold in association with hyperplasia in 2 of the TGM. Cr overexpression was consistently observed in hyperplastic mammary glands in all the animal models, decreasing in overt tumors in 2 of the TGM models. In MMTV-PyMT mammary glands, the levels of Cr expression rose 7- to 10-fold in hyperplastic tissue and 25-fold the levels in tumors compared to age-matched transgene negative mice. Ar and especially Cr thus should have potential value as markers of preneoplastic change in mammary tissue. *Int. J. Cancer* 81:588–591, 1999.
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A major goal of cancer research is to identify transforming oncogenes and determine their precise role in tumor development. Genes such as Amphiregulin (*Ar*), transforming growth factor alpha (*TGFα*), Cripto (also known as *TDGF-1*), epidermal growth factor receptor (*EGFR*) and *ErbB2/neu* are over-expressed in and/or are markers for human breast carcinomas. Invasive mammary carcinomas generally exhibit increased levels of Ar protein and/or mRNA compared to ductal carcinomas *in situ* or normal mammary epithelium. Ar, one of several ligands for EGFR, is believed to act as a mediator of proliferation and/or differentiation via an autocrine mechanism (Salomon *et al.,* 1995) . Of 68 biopsies of breast carcinomas, 82% expressed CR (Qi *et al.,* 1994). CR (receptor not known) expression was not detected in normal human breast tissue or cell lines, and its appearance was thought to be associated with transformation. We have shown that Cr is highly expressed during the pregnancy and lactation stages of normal mouse breast development acting as both a proliferation and survival factor (Kenney *et al.,* 1995; Niemeyer *et al.,* 1998). About 40% of breast tumors over-express epidermal growth factor receptor (EGFR) (Klijn *et al.,* 1992). EGFR is a transmembrane inserted tyrosine kinase protein with a Mr of approximately 170,000 (Cohen *et al.,* 1982) that can be activated by the ligands EGF, TGFα, betacellulin and epiregulin in addition to Ar. The second member of the ErbB family of receptor genes, *ErbB2* gene (*HER2, c-neu*), encodes an Mr 185,000 transmembrane tyrosine kinase protein (King *et al.,* 1985; Schechter *et al.,* 1984). Amplification and overexpression of ErbB2 has been observed in nearly 30% of human cancers, particularly intraductal carcinomas (Hynes and Stern, 1994).

In this study we used transgenic mice overexpressing PyMT, erbB2, or TGFα in the mammary glands (Smith *et al.,* 1995; Jhappan *et al.,* 1990; Guy *et al.,* 1992*a*,*b*) to study the expression patterns of 2 recently discovered growth factor breast cancer markers, Ar and Cr. Our present results indicate that Ar and Cr could be useful as early markers of hyperplastic growth that precede the onset of tumors.

MATERIAL AND METHODS

Western blot analysis

Mammary tumors and tissues were obtained from staged mice. The tissues were homogenized in hypotonic buffer (20 mM HEPES, pH 7.4; 1 mM EDTA; 1 mM $MgCl_2$; 1 µg/ml phenylmethylsulfonyl fluoride; and 20 µg/ml aprotinin) and solubilized in Laemmli sample buffer. Equal amounts of protein were electrophoresed on a 15% SDS-PAGE gel and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed and visualized using the ECL detection system (Amersham Corp., Aylesbury, UK).

Antibodies

The Cr antibody used in this study consisted of a rabbit polyclonal raised against a murine Cripto peptide, amino acid sequence 26 to 39, RDLAIRDNSIWDQK. The use and validation of this antibody was previously described (Niemeyer *et al.,* 1998). The amphiregulin antibody used in this study, AB2426, was a rabbit polyclonal raised against a synthetic peptide corresponding to the murine amphiregulin sequence 119 to 134, RKKKGGKNGKGRRNKK. A similar peptide antibody (AR-Ab2) was characterized in colon carcinomas (Johnson *et al.,* 1992). The ErbB2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and sheep anti-human EGFR intracellular domain was from GIBCO-BRL (Gaithersburg, MD).

Transgenic mice

The PyMT mice (in an FVB/N background) have been fully described (Guy *et al.,* 1992*a*). MMTV-neu mice (JR2376; FVB/N-TgN(MMTVneu)202Mul) were obtained as homozygotes also in an FVB/N background from the Jackson Laboratory, Bar Harbor, ME (Guy *et al.,* 1992*b*). MT-TGFα transgenic mice were described earlier (Smith *et al.,* 1995; Jhappan *et al.,* 1990). Transgenic lines were maintained in our Institute Animal Facility by mating founder/positive animals to littermates. Transgenic mice were identified by polymerase chain reaction (PCR) analysis of tail DNA using oligonucleotide primers specific for the transgene. For each stage, the left 4th inguinal mammary gland was dissected out for immunoblotting and the right gland was stained as a whole mount, to indicate the tumor status by gross morphology. Hyperplasia was recognized by numerous small multifocal H & E-staining islands of ductal or lobular epithelial cell proliferation, occupying 20% of the total gland. Larger dense aggregates of cells were designated tumors, of which the largest were palpable. Two or 3 samples were used for each data point, except for MT-TGFα mice where there were 2 tumors and one of each of the other samples.

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RESULTS

MMTV-PyMT transgenic mice (TGM)

PyMT overexpressing FVB/N TGM express the oncogenic polyoma virus middle T antigen from the MMTV promoter/enhancer. Extensive multifocal transformation of the mammary epithelium ensues and rapidly progresses to mammary adenocarcinomas (Guy *et al.*, 1992*a*). Hyperplasias start to appear on the 32nd day in females, and therefore mammary glands from virgin females were examined at postnatal days 20, 28, 32, 44 and 56. We studied 2–3 animals per sample point and found no palpable tumors up to day 44, but by day 56, palpable tumors were present. Immunoblot analyses to detect and quantify the levels of Ar and Cr proteins in mammary tissues together with EGFR and ErbB2 were performed (Fig. 1). Densitometry measurements of the results were normalized for the protein content by the signal obtained from either β-actin or α-actinin on all analyses. The highest level of each gene product was given a score of 100% and the other samples were compared to this level (Table I). For comparison, normal tissues and tumors were retrieved from both transgene negative and positive 8-week virgin mice, respectively; 13.5 day pregnant mice and 1 mouse that was 1 day post partum and was not able to lactate.

Amphiregulin was expressed in the mice positive for the PyMT transgene (+ in Fig. 1) in mice 28- and 32-day-old but not in normal littermates (-) at these timepoints. Interestingly, at day 20, when sex steroid hormones were being activated at the onset of puberty, Ar expression was observed in both positive and negative mice, and was 1.5-fold higher in the transgene positive mice. By day 44, hyperplasia and scattered non-palpable dense aggregates were observed (data not shown) and Ar levels increased significantly in this tissue (Fig. 1, lane 10). Ar levels were increased 10-fold in hyperplastic glands and up to 25-fold higher in tumors of 56-day virgin mouse mammary glands (Table I). However, there was no significant difference between the Ar expressed by pregnant Tg^+ or Tg^- mice or by lactating mice. These hormone-induced levels may be the highest possible that can be expressed by the mammary gland and were given a score of 100% in Table I. Among the different isoforms of Ar that were seen in these studies, only a large doublet of 28 and 25 kDa were produced by the PyMT TGM mammary glands prior to pregnancy.

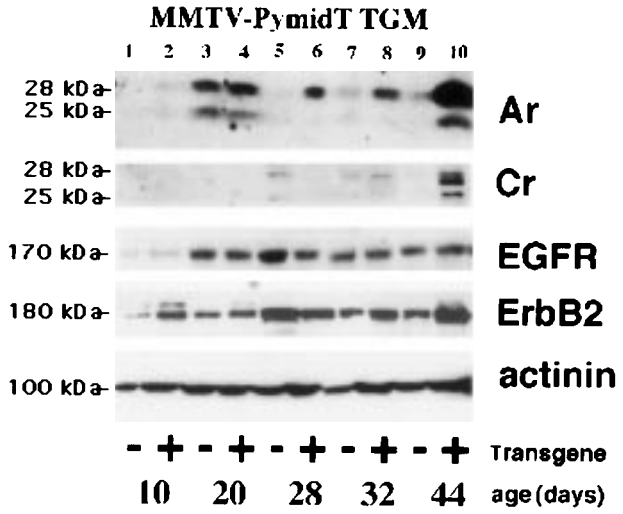

FIGURE 1 – Expression analysis of proteins in mammary gland tissues and tumors derived from MMTV-PyMT transgenic mice by immunoblotting. No palpable tumors were present in these tissues. Note that the sample 44+ was hyperplastic with small non-palpable tumors. The (+) or (−) refers to the presence or absence of the transgene. In some analyses α-actinin was used as a loading control, in others, β-actin served this function.

Using the same lysates, no Cr expression was observed until day 32 and these were very low levels that were no greater than in the control (−) mouse. We have observed before that Cr is expressed at very low but detectable levels in older virgin mice (Fig.1, lanes 5, 7 and 8) (Niemeyer *et al.*, 1998). By day 44 in virgin females, Cr levels were 7- to 10-fold higher than in control (−) mice (Fig. 1, lane 10). At 56 days of age, the tumors from virgin mice showed 25-fold higher levels of Cr than the transgene-negative samples (data not shown). The tumors in pregnant and lactating mice expressed high levels of Cr that were not significantly different from the levels of Cr expressed in the non-transgenic mice (Table I). This is probably due to the increased levels of prolactin and glucocorticoids that upregulate Cr (and Ar) during pregnancy (Niemeyer *et al.*, 1998). Several isoforms of Cr were observed (28, 27 and 25 kDa), probably caused by differential post-translational modifications and by degradation in larger tumors that were necrotic and have described previously by others (Brandt *et al.*, 1994; Kenney *et al.*, 1995, 1996b).

The levels of EGFR protein expressed in all of the samples derived from PyMT positive and normal mammary glands were low and unchanging through the progressive series except for a slight up-regulation at puberty (Fig. 1, lanes 3 to 6). No differences were observed between tumors derived from virgin, pregnant and lactating mammary glands**.** ErbB2 protein was observed in all of the MMTV-PyMT mammary glands and tumor samples assayed, but there was no significant difference between mice that were positive for MMTV-PyMT and normal mice until the time that hyperplasia was seen (Fig. 1, lanes 9, 10). Thereafter tumors expressed 2- to 3-fold more ErbB2 than their non-transgenic sisters (Table I).

TABLE I – SUMMARY OF PROTEIN EXPRESSION LEVELS IN MAMMARY GLANDS AND TUMORS OF TRANSGENIC MICE[1]

MMTV-PymidT	20 day virgin	Pre-hyperplastic	Hyper-plasia	Tumor	Pregnant	Lactating
Ar						
28 kDa	+	+	++	+++	++++	+++
25 kDa	+/−	+/−	+	+	+	+
15 kDa	−	−	−	+/−	++++	+++
Cr						
28 kDa	−	+/−	++	++++	++++	+++
25 kDa	−	+/−	++	++++	+++	++
EGFR	+++	+++	+++	++++	+++	+++
ErbB2	++	+++	+++	++++	+++	+++

MMTV-ErbB2/neu	Pre-hyperplastic	Hyperplasia	Tumor	Male
Ar				
28 kDa	−	−	++++	−
25 kDa	−	−	++++	−
15 kDa	+/−	+/−	−	+++
Cr				
28 kDa	+/−	++++	+/−	+
EGFR	++	++	++++	+++
ErbB2	++	++	++++	+

MT-TGFα	20 weeks	Pre-hyper-plastic 8 month	Hyper-plasia	Adeno-Ca	Ade-noma	Pregnant
Ar						
25 kDa	+/−	++++	++++	++	−	+
15 kDa	+/−	++	++	++	−	++++
Cr						
28 kDa	+/−	+/−	++++	++	−	++++
EGFR	++++	++++	+++	+++	++	++++
ErbB2	+++	+++	+++	++++	+/−	+++

[1]At least 2 separate tissue samples were analyzed and the results averaged. ++++, 75–100% (highest level of expression of given protein per tumor system); +++, 50–75%; ++, 25–50%; +, 10–25%; +/−, 1–10%; −, background.

MMTV-ErbB2 transgenic mice

Overexpression of the normal *c-ErbB2* gene gives rise to mammary tumors with a long latency (Guy *et al.*, 1992*b*). According to Guy *et al*. (1992*b*), tumors first appear as foci in surrounding hyperplastic growth at or after 4 months of age with a median incidence of 205 days. In these analyses, we were able to collect 2 samples each of hyperplastic glands and 2 overt tumors with their surrounding tissues to analyze for gene expression. Ar was expressed as a large 25–28 kDa species in tumor tissue at much higher levels than in the surrounding tissue but was not overexpressed in precancerous tissue, in contrast to Ar expression in PyMT mice (Fig. 2 and Table I). Another isoform of 14–16 kDa was expressed in normal and tumorous tissues and this increased with age and was the most prevalent isoform in male mammary gland tissue (Fig. 2).

Cr, on the other hand, was strongly expressed in 20-week-old virgin female mammary glands from MMTV-ErbB2 mice that had hyperplastic growth (Fig. 2, lane 2), whereas earlier stages were negative. In the overt tumors in this mouse, however, little if any was expressed, while Cr was present at low levels in the adjacent tissue. This result is in contrast with the results in MMTV-PyMT mice in which Cr was expressed at greater levels in the tumor itself than in the surrounding tissues and less in the hyperplastic, precancerous gland. In the c-ErbB2 mammary tumor model, Cr is a clear marker for hyperplasia of the mammary gland.

As expected, in MMTV-ErbB2 mice, the levels of ErbB2 expressed were much greater in tumor tissues than in the hyperplastic or surrounding ones. In addition, EGFR levels were also much higher in the tumors of these mice than in the surrounding or hyperplastic tissues, suggesting that EGFR is co-induced in these transgenic mice.

MT-hTGFα transgenic mice

The hTGFα-expressing virgin mice do not develop tumors until after approximately 8–9 months, and after multiple pregnancies. Several isoforms of Ar were observed in these samples. A doublet of approximately 26 kDa was observed as well as a 14 kDa doublet. The higher 26 kDa doublet showed greatest levels of expression in the 8 month transgene positive virgin mammary tissue (Fig. 3, lane 1). Lower levels of expression were observed in the hyperplastic and adenocarcinoma tissues as well as in normal transgene negative pregnant mice. No expression of this doublet was observed in the adenoma or normal male samples or in 8 and 20 week virgin transgene positive mice (data not shown). Of more interest, the higher m.w. band of the 14 kDa doublet showed greatest levels in hyperplastic and pregnant tissues suggesting that this isoform is expressed in proliferative mammary tissue. The lower m.w. band showed highest expression in virgin, adenocarcinoma and pregnant tissues.

In the hTGFα expressing mice (Fig. 3), Cr levels were high in the samples from breast hyperplastic tissue. This high level was reached only in the mammary glands of normal pregnant mice. Interestingly, only an intermediate level of Cr remained in the adenocarcinoma samples, while little or none was seen in the adenoma. Cr expression was thus correlated with highly proliferative mammary cells.

EGFR expression was high through 8 months of age (Fig. 3, lane 1). Approximately the same levels were found in the pregnant mammary gland tissue. Slightly less EGFR protein was observed in hyperplasias and adenocarcinomas and significantly less was observed in the adenomas (Fig. 3, lane 4). Interestingly, no ErbB2 was observed in adenomas and normal male mammary glands, while moderate levels occurred in virgin and pregnant mice, and this increased by 2- to 3- fold in adenocarcinomas (Fig. 3, lane 3).

DISCUSSION

Mammary tumor progression cannot be readily analyzed in humans and therefore the transgenic mouse offers the most suitable model to study putative tumor marker expression preceding and during the formation of tumors. Tissue from a single line of mice allows analysis of tumor stages that are fairly consistent in time of progression up to overt tumor formation. We selected Cr, Ar, EGFR and ErbB2 to study because of their close association with mammary tumors and because these genes are also expressed in normal mammary gland development. Each ligand of the EGFR can up-regulate both the receptor and the other ligands thus increasing the probability of progressive loss of growth control (Barnard *et al.*, 1994). The PyMT-expressing mice develop hyperplasias and tumors the most rapidly of the 3 models examined,

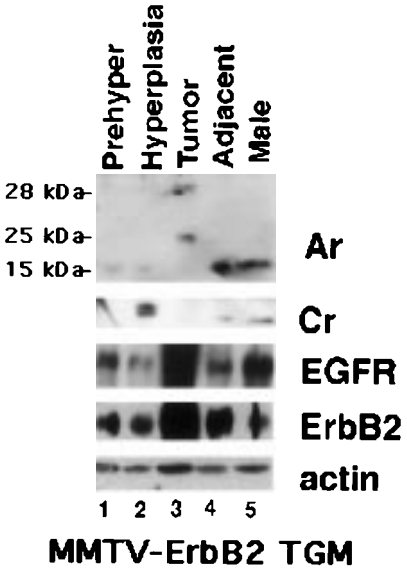

FIGURE 2 – A similar immunoblot analysis of mammary tissues from MMTV-ErbB2 transgenic mice. Four stages are shown and compared to the levels of protein expressed in male mammary gland (lane 5). All the samples came from transgene positive mice.

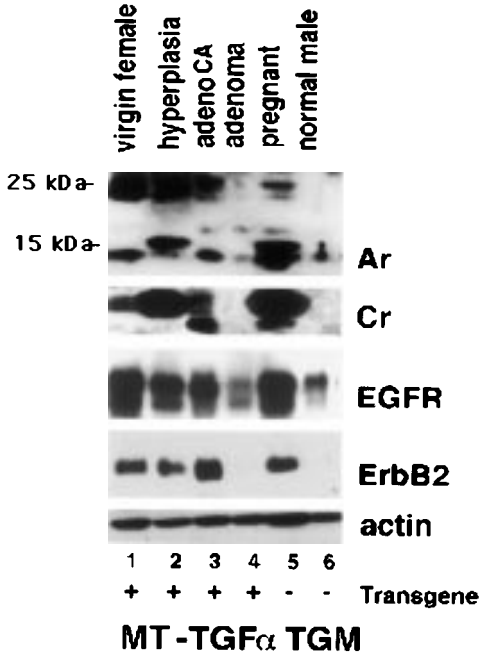

FIGURE 3 – A similar immunoblot analysis of the mammary tissues derived from MT-TGFα transgenic mice. Malignant adenocarcinoma tissue is shown in lane 3 and benign adenoma tissue in lane 4.

placing these transgenic mice at one extreme of the tumorigenic process. However, the PyMT is a viral gene that is not expressed in the human breast, whereas TGFα and ErbB2 genes are normal gene products overexpressed in human tumor cells, and therefore, these transgenic mouse models are more likely to be closest to the human disease.

We report here that Cr is an early marker for pre-tumoral/hyperplastic tissue in all 3 transgenic models examined. Cripto levels are significantly higher in the tumors themselves formed by overexpression of the polyoma virus middle T antigen compared to tumors caused by ErbB2 or TGFa overexpression. This finding contrasts with those of Kenney *et al.* (1996*a*,*b*), who found that mammary tumors in the same MMTV-transgenic mouse strains all expressed the highest levels of Cripto and amphiregulin. However, they did not examine premalignant stages. Cr is a proliferative factor for the mammary gland but does not itself appear to cause tumors in mammary cells (Niemeyer *et al.*, 1998). Cr is upregulated during hyperplastic growth in pre-tumoral mammary glands, as well as in normal pregnancy. It remains to be determined whether Cr is a suitable marker for early precancerous breast hyperplasia in human patients.

Amphiregulin is a bifunctional polypeptide growth regulator that has been demonstrated to bind solely to the EGFR and induce *in vitro* tyrosine phosphorylation of this receptor in human mammary and ovarian cell lines (Johnson *et al.*, 1993). Ar is a known autocrine growth factor for the mammary gland and does appear to cause hyperplastic growth in normal mammary glands (Kenney *et al.*, 1996*b*) but these do not develop further into overt tumors. While Ar becomes overexpressed in hyperplastic MMTV-PyMT mammary tissue and hyperplastic MT-TGFα mammary tissue, we found that the overexpression of ErbB2 stimulates Ar overexpression only at the overt tumor stage**.** A normal human mammary cell line overexpressing c-ErbB2 (MCF-10A neu cells) displays a 15 to 30-fold increase in the *in vitro* levels of Ar mRNA and protein (Normanno *et al.*, 1994) compared to parental cells. c-ErbB2 is a marker for some tumors and its overexpression is correlated with poor prognosis. Based on this evidence, ErbB2 expression may be regarded as a late stage marker in the progression to malignant disease. As observed with Cripto expression, of the 3 tumor models, the tumors due to polyoma virus middle T antigen expression exhibited the highest levels of Ar.

We have shown that Cripto is a proliferative factor that is overexpressed during hyperplastic growth in all mouse mammary tumor models studied. Amphiregulin was overexpressed in 2 of 3 models of hyperplasia. For both genes, the mammary gland in pregnant mice achieved the same high levels of expression, but this is unlikely to interfere with the usefulness of Ar and/or Cr as a marker(s) of the hyperplastic mammary gland.

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