Characterization and chromosomal instability of novel derived cell lines from a wt-erbB-2 transgenic mouse model

Jacqueline S.Jeruss1-2, Na Xin Liu2, Yongji Chung2, Gregg Magrane3, Frederic Waldman2, Susan Edgerton4, Xiaoye Yang3 and Ann D.Thor1,5

1Department of Pathology and Department of Surgery, Northwestern University School of Medicine, Chicago, IL; 2Evanston Northwestern Healthcare Research Institute, Evanston, IL; 3Molecular Cytogenetics Core, University of California at San Francisco/Mount Zion Cancer Center, San Francisco, CA and 4Department of Pathology, Oklahoma University Health Sciences Center, 940 Stanton L.Young Boulevard, Room 451, Oklahoma City, OK 73104, USA
5To whom correspondence should be addressed
Email: ann-thor@ouhsc.edu

Introduction

Breast cancer is a highly morbid and prevalent disease associated with a wide variety of risk factors, of which genetic predisposition and estrogen (E2) exposure are paramount. Some breast cancer risk factors are independently associated with patient prognosis (1). One such risk factor, the erbB-2 (HER-2/neu) proto-oncogene, is located on human chromosome 17q21 (2). The erbB-2 gene encodes an orphan receptor transmembrane protein, which has intrinsic tyrosine kinase activity, and is a member of the receptor tyrosine kinase type I epidermal growth factor receptor (EGFR) superfamily (3,4). Members of the EGFR superfamily influence cell cycle progression and cellular proliferation (5–7). The erbB-2 gene is commonly amplified and the protein p185 is overexpressed in human breast cancer (8).

The hormone E2 is a known mammary epithelial cell carcinogen (9). The carcinogenic effects of E2 vary with patient age, dose and duration of E2 exposure (10,11). Estrogenic control over several G1 cell cycle regulators and growth factors has been demonstrated both in vivo and in vitro (12,13). Estrogenic promotion of cell proliferation in particular is believed to put responsive cells at risk for carcinogenesis by facilitating or even inducing the acquisition of genetic changes during cell cycle progression (13,14). The clinical prevention of breast cancer by the anti-estrogen tamoxifen serves as proof of principle for this concept (15,16). Endogenous modifiers of hormonal levels include age, body habitus and physical activity. Exogenous factors, such as plant-derived steroids (phytoestrogens), may also modify hormonal levels and breast cancer risk (11,17). Pre- and peri-pubertal exposure to a diet high in soy has been shown to decrease mammary carcinogenesis in both rat model systems and in humans (11,18,19). The phytoestrogens most prevalent in soy, daidzein and genistein, are capable of binding to the estrogen receptor and are selective estrogen receptor modulators (20).

To study the effects of estrogen exposure on genetically at risk virgin transgenic mice bearing the wild-type rat erbB-2 gene, under control of the MMTV promoter (MMTV-neu), we used dietary soy (as compared with a control casein diet) or short-term exposure to a continuous release estradiol (E2) or placebo pellet implanted at 8 weeks of age. Using this well-characterized transgenic model (21,22), we have now demonstrated mammary tumor induction at a younger age post-E2 exposure and prolongation of latency by a diet rich in soy meal (X.H.Yang, S.M.Edgerton, S.D.Kosanke, T.L.Mason, K.M.Alvarez, N.Liu, R.T.Chatterton, S.M.Murthy and A.D.Thor, submitted). E2 exposure was also associated with the development of more aggressive tumors, identified by higher histological tumor grade, multifocal (as compared with unifocal) tumor development, extensive stromal invasion (as contrasted with nodular tumor growth) and more frequent pulmonary metastasis. We have also recently demonstrated that short-term tamoxifen-exposed mice generally failed
identifiable tumors were either snap frozen, formalin fixed and paraffin embedded and the age of tumor development. Mammary glands from each treatment group were used for characterization by CGH and additional methods.

We originally hypothesized that E2 exposure would be associated with enhanced chromosomal changes and that a soy diet might reduce this association. We sought to determine if: (i) chromosomal alterations were present in parental tumors or derived cell lines; (ii) heterogeneous (random) or selected chromosomal instability would occur; (iii) chromosomal instability was specifically affected by treatment or dietary grouping for either the tumors or derived cell lines.

Materials and methods

Estrogenic exposure studies by dietary group

For cell line and growth characteristics studies, 70–5-week-old female FVB/N-Tg (MMTV-neu) mice (Jackson Laboratories, Bar Harbor, ME) were divided into two separate groups and fed either institutional standard animal chow (based on soy protein, Purina 5001; Ralston Purina, St Louis, MO) or a similar casein-based rodent chow (Purina 5K96) (26). Animals were maintained on either of these diets for the duration of the project. At 8 weeks of age, 19 animals on Purina 5001 and 20 animals on Purina 5K96 had s.c. implants of a 0.5 mg (~200 g/ml) 60-day ‘constant’ release E2 pellet placed in the lateral neck (Innovative Research of America, Sarasota, FL). Control female FVB/N-Tg (MMTV-neu) mice (Jackson Laboratories, Bar Harbor, ME) were given a placebo pellet.

Animals were killed at 15, 20, 25, 30, 35, 55 and 59 weeks of age (or when a palpable tumor reached 1.2 cm in diameter). The age of tumor detection by palpation was recorded as the age of tumor development. Mammary glands were surgically removed and processed immediately following killing. Grossly identifiable tumors were either snap frozen, formalin fixed and paraffin embedded or minced to provide cells for primary tissue culture. Over 33 novel, primary cultured cell lines derived from the mouse mammary tumors have been developed. All cell lines have been passaged repeatedly and are believed to represent stable cell lines in culture. Several stable lines selected from each treatment group were used for characterization by CGH and additional methods in vitro.

Establishment of cell lines

To establish mammary tumor cell lines from the transgenic mice, tumor tissue was surgically resected and immediately processed. The tumor tissue was minced with scissors and trypsinized (25% trypsin and 1 mm EDTA; Invitrogen Corp., Grand Island, NY) for 30 min at 37 °C. The tissue fragments were then re-minced with scissors and placed in a test tube with DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G + 10 μg/ml streptomycin (Invitrogen Corp.). Fine tissue explants were cultured in DMEM/F12 medium supplemented with 10% FBS and penicillin + streptomycin. Cells were counted using a hemocytometer and then plated at 1 × 10^4 per 60 mm plate. Cells were passaged until stability was achieved. Long-term storage was provided through freezing in liquid nitrogen. Using these methods we have developed over 33 novel mammary tumor-based cell lines. Representative cell lines were randomly selected for analysis from our diet/treatment groups after stabilization. Initial characterization of the cell lines included cytokeratin and vimentin staining and flow cytometry.

Flow cytometry

Flow cytometry was performed to determine cell cycle distribution and cell ploidy. Trypsinized cells were washed once in phosphate-buffered saline (PBS) without Ca^2+/Mg^2+ and, after resuspension in 500 μl PBS, cells were added drop-wise into ice-cold 70% ethanol. Fixed cells were then resuspended, counted and aliquots of 3–5 × 10^5 cells were placed in flow cytometry tubes. Cells were resuspended in PBS, washed, centrifuged and resuspended in PBS. Cells were then fixed in ethanol and stored at −20 °C until use. For flow cytometry, cells were resuspended in PBS with 1% BSA and 0.1% Triton X-100 for a final wash. Cell pellets were then resuspended in 900 μl of 50 mM sodium citrate buffer, pH 7.8, containing 50 μg/ml propidium iodide and 50 U/ml RNase A (Sigma Aldrich Co., St Louis, MO). Tubes were incubated at 37 °C in the dark for 45 min. Cell cycle analysis was performed using a FACSCalibur system (Becton Dickinson, San Jose, CA). The fractions of the total cell population present in each of the G1, S and G2/M phases and ploidy were determined from DNA histograms by mathematical modeling using MultiFit software (Verity Software House, Topsham, ME).

CGH

CGH was performed as described previously (27), using DNA isolated from frozen primary tumors and derived cell lines generated from the four treatment groups. The Wizard™ Genomic DNA extraction kit (Promega UK) was used to isolate tumor DNA. Tumor and cell line DNA and normal reference DNA (labeled with distinct fluorescent dyes) were hybridized simultaneously onto normal C57 Black mouse metaphase chromosomes generated from embryo fibroblasts. The ratio of tumor to reference fluorescence intensities was used as a measure of gains and losses of DNA localized to specific chromosomal regions. Each sample was hybridized in duplicate. Successful hybridizations were judged by the intensity of the tumor and normal signals, by the granularity and smoothness of the signals, by the homogeneity of the signal over the entire metaphase and by the banding intensity of the 4',6-diamidino-2-phenylindole signals used for chromosome identification. Image acquisition of the metaphase spreads was performed using the Powergene MacProbe Imaging System (Applied Imaging, Santa Clara, CA).

Chromosomal gain or loss were defined as chromosome regions that had a test to reference ratio > 1.2 or < 0.8. The duplicate inversely labeled pair was examined together to allow better discrimination of significant changes. Significant changes were defined as changes seen in both the forward and inverse hybridizations (28).

Doubling time in culture

Measurement of growth rates in culture (in a monolayer) were determined using sulforhodamine B (SRB) assays as previously described (29). Two thousand cells were seeded into each well of a 96-well plate with complete medium. Cells were fixed with 10% trichloroacetic acid (TCA) every 24 h for 6 days after inoculation. TCA fixed cells were then stained with 0.4% SRB for 30 min followed by four washes. Protein-bound dye was dissolved in 10 mM Tris base and plates were read at 565 nm using an ELISA reader. Cell doubling time was calculated based on proliferation curves resulting from the change in SRB absorbance over time.

Soft agar cloning assays

Soft agar cloning was performed using a previously reported methodology (30). A 30% agarose stock was heated to 50°C. A slurry of 100 ml of supplemented medium (complete medium) was mixed with 8 ml of the agarose stock and maintained at 42°C. From this, 4 ml was pipetted into 60 mm culture dishes and allowed to set for 20 min. Twenty thousand cells (0.8–1.0 ml of complete medium) were added in a drop-wise fashion to each culture dish (in triplicate per cell line). After sitting for 10 min, the culture dishes were placed at 37°C incubator. Cultures were fed weekly and counted using an inverted microscope after 3 weeks. Colony counts were obtained from three dishes per cell line counting all colonies > 50 μm in diameter.

Statistics

Comparisons of numbers of tumors per mouse between groups (soy versus casein, E2 versus placebo) were calculated using the two-tailed Student’s t-test. Tumor latency between groups on the same diet or between diets was compared using the Cox’s proportional hazards model using the logrank statistic (31). The first palpable tumor was used to calculate the tumor latency for animals that had palpable tumors. The date of killing was used to calculate tumor latency for animals without palpable tumors. Animals killed without tumors were censored at the date of death for tumor-free survival analysis and incidence analysis. Censoring was necessary because animals killed early in the study (used to compare morphological and histological differences between groups or diets) had not developed tumors by the date of death. In addition, animals found dead and not necropsied were also censored for incidence or survival analysis. Statistical calculations were carried out using Stat View 5.01 software (SAS Institute, Cary, NC).

Results

Mammary gland tumors and derived cell lines from virgin, transgenic mice bearing the wild-type rat erbB-2 gene under the control of the MMTV promoter initially developed by Muller in 1991 (21) were used for this study. Mice were...
divided into four diet/treatment groups: soy meal diet (Purina 5001)/E2-treated; casein diet (Purina 596K)/E2-treated; soy diet/placebo pellet-treated; casein diet/placebo-treated. The \textit{in vivo} effects of diet and E2 exposure on this model system by diet are summarized by a plot of tumor incidence by age (Figure 1). A brief exposure to E2, beginning at 8 weeks of age, shortened tumor latency for mice fed either diet. Soy meal chow-fed mice experienced a longer tumor-free interval in both the E2 treatment and placebo pellet-treated groups.

\textbf{Characterization of the derived lines by CGH}

Cell lines were derived from mammary tumors that formed spontaneously in the transgenic mice. Approximately four stable cell lines were randomly selected from each treatment group for this study. The derived lines displayed epithelial morphology and polygonal cell shapes (representative morphology shown, Figure 2). Many cell lines also displayed pseudo-gland formation. Table I lists the cell lines and corresponding mammary tumors analyzed by CGH for each group. The average instability (gains and losses) was 2.75 for the E2/soy cell lines, 1.4 for the E2/casein group, 1.97 for the placebo/soy group and 1.5 for the placebo/casein group. Hence, the E2/soy group demonstrated the greatest genomic instability by CGH in this random sampling. Cell lines derived from soy-fed mice exhibited greater instability than their casein-fed, matched treatment groups.

The most common chromosomal abnormality in all the lines was a complete loss of mouse chromosome 4, occurring in 7/16 (43.8%). In one line, the loss was limited to chromosome 4D-E. The second most frequent change was a gain of chromosome 10, occurring in 3/16 (18.7%) of the lines. Heterogeneity in the total number and location of gains and losses existed between all cell lines. In order to determine if the CGH defined instability was acquired during cell culture or inherent in the primary mammary gland tumors, at least two corresponding parental tumors per group were also analyzed by CGH. As shown in Table I, tumors from the E2/soy, E2/casein and placebo/soy groups showed no gains or losses by CGH. In tumors from the placebo/casein group, 2/2 demonstrated complete loss of chromosome 4. These data are consistent with the acquisition of genetic change with cell line passage and clonal evolution.

A minimum of three cell lines from each group were then analyzed \textit{in vitro} by CGH and other assays of aggressive behavior. Cell lines derived from the E2/casein mice demonstrated the most aggressive \textit{in vitro} phenotype with the highest colony formation efficiency in soft agar (an average of 26.4 colonies), over 30% of cells in S phase by flow cytometry and the most rapid doubling time (Table II). These lines were derived from mammary tumors that arose with the shortest latency and were more aggressive \textit{in vivo} (significantly higher histological grade, more often invasive stroma, metastastic, etc.; 23,24) Of interest, these lines demonstrated the fewest chromosomal gains and losses (see Tables I and II). Other groups demonstrated less aggressive \textit{in vitro} characteristics, which also corresponded to a less aggressive \textit{in vivo} phenotype. This was true despite higher average numbers of chromosomal gains and losses in these groups, particularly in the soy-fed mice.

\textbf{Discussion}

The genetic diversity of human breast cancer cell lines has been well described (32). Cell lines derived from transgenic mouse mammary gland tumors are rare, with a single previously established cell line reported derived from this wt-erbB-2 transgenic model (33). Genetic instability in derived lines is not often studied, although Montagna \textit{et al.} have recently reported several derived cell lines from the mutant erbB-2 transgenic mammary tumor model (34). Various methods have been used to explore genetic and phenotypic changes in tumors and derived cell lines and to determine if differences in treatment/diet groups could be associated with specific biological features. Heterogeneity in the total number and location of chromosomal gains and losses was observed in the mammary tumor-derived cell lines and, to a lesser extent, in the parental mammary tumors. Nearly half of the lines showed a loss of chromosome 4. Lines derived from tumors...
that arose in soy-fed mice exhibited greater chromosomal instability, with the E2/soy group showing the most genetic change. We hypothesize that these lines were more permissive to, or less well able to repair, genetic alterations that occurred with the selection pressure of primary cell culture and passage. Parental tumors used to generate these lines developed with a longer latency than treatment matched casein-fed mice. Tumors from the E2/casein tumor group appeared to be more aggressive both in vivo and in vitro. Many studies have demonstrated an association between latency and the amount of genetic change consistent with a multi-hit process (35,36). Because CGH cannot detect small changes like point mutations, we hypothesize that genomic alterations not identified by CGH may be present in those tumors that occur with longer latency (like p53 point mutations) that might facilitate this observation. We did not observe an association between either E2 or phenotypic aggression and the amount of major genetic change identified by CGH. Nonetheless, there was an association between shortened latency in vivo and tumor aggression. A similar association has been observed for breast cancers that arise with shorter latency in young women (37).

The CGH findings in this study parallel the recently reported work of Montagna et al., who utilized CGH and spectral karyotyping to examine cytogenetic abnormalities in an oncogenic (mutant) erbB-2/neu transgenic mouse model (34). DNA from the mutant erbB-2 mouse mammary tumor-derived cells also exhibited chromosome 4 abnormalities in conjunction with genomic amplification of erbB-2. Specifically, in the mutant erbB-2 transgenic study, erbB-2 was amplified in the form of double minute chromosomes, and the loss of mouse chromosome 4D-E was also frequently noted (34). In contrast, we observed only a single line with an isolated loss of chromosome 4D-E and generalized loss of chromosome 4 in many lines and two parental tumors. The relatively common loss of mouse chromosome 4 in erbB-2-associated mammary tumors, either of the wild-type (as we identified) or from mutated erbB-2 transgenic models (as identified by Montagna et al.), is of interest. Non-random genetic alterations found in human

### Table I. CGH alterations in parental mammary tumors and derived cell lines

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cell line</th>
<th>Tumors</th>
<th>Gains</th>
<th>Losses</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/soy</td>
<td>1</td>
<td>None</td>
<td>6, 8, 16</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>13, 19</td>
<td>2, 3, 4, 7, 11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Mean no. of changes</strong></td>
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<td><strong>1</strong></td>
<td>1.25</td>
<td>1.5</td>
</tr>
<tr>
<td>E2/casein</td>
<td>1</td>
<td>None</td>
<td>18</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>10, 15, X (A-B)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>None</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>None</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Mean no. of changes</strong></td>
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<td><strong>1</strong></td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Placebo/soy</td>
<td>1</td>
<td>None</td>
<td>2, 11, 12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>10</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>None</td>
<td>18</td>
<td>None</td>
</tr>
<tr>
<td><strong>Mean no. of changes</strong></td>
<td><strong>1.97</strong></td>
<td><strong>1</strong></td>
<td>1.67</td>
<td>0.3</td>
</tr>
<tr>
<td>Placebo/casein</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>15</td>
<td>2, 4 (D-E)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>3, 4</td>
</tr>
<tr>
<td><strong>Mean no. of changes</strong></td>
<td><strong>1.5</strong></td>
<td><strong>1</strong></td>
<td>0.25 gains</td>
<td>1.25 losses</td>
</tr>
<tr>
<td>Placebo/casein</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><strong>Mean no. of changes</strong></td>
<td><strong>1.5</strong></td>
<td><strong>1</strong></td>
<td>0.25 gains</td>
<td>1.25 losses</td>
</tr>
</tbody>
</table>

*aCases 1 and 2 from the cell lines were derived from tumors 1 and 2 for each group.*

### Table II. In vitro data by treatment and diet group

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Doubling time (h)</th>
<th>Colonies in soft agar</th>
<th>S phase by FACS</th>
<th>Average G/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/soy</td>
<td>3</td>
<td>24.2</td>
<td>14.1</td>
<td>17.0</td>
<td>2.75</td>
</tr>
<tr>
<td>E2/casein</td>
<td>3</td>
<td>21.1</td>
<td>26.4</td>
<td>33.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Placebo/soy</td>
<td>3</td>
<td>24.8</td>
<td>16.7</td>
<td>34.1</td>
<td>1.97</td>
</tr>
<tr>
<td>Placebo/casein</td>
<td>3</td>
<td>24.4</td>
<td>17.3</td>
<td>21.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*n, number of cell lines; FACS, fluorescence-activated cell sorting analysis; G/L, gains/losses.*
breast cancers showing erbB-2 amplification have been studied using CGH techniques (38). erbB-2 amplified tumors typically demonstrate twice the number of genomic abnormalities by CGH as compared with tumors without amplification (38). In human mammary cancers, somatic alterations in erbB-2-altered breast cancers include frequent gains of chromosomes 1q, 8q and 20q and losses of 18q, 13q and 3p (38). Thus, human breast cancers with erbB-2 abnormalities do not commonly demonstrate alterations of chromosome 1p, the orthologous region to mouse chromosome 4D-E (www.informatics.jax.org/reports/homologymap/mouse_human.shtml). While a link between the loss of potential tumor suppressor genes located on chromosome 4 and tumorigenesis in the erbB-2 transgenic mouse model system is possible, the mechanistic associations with specific genes are as yet undefined. Even if a linkage is identified in transgenic mice, it may not correlate with a mechanism of tumorigenesis in human breast cancer (34,39).

An interesting feature of this model system is the plasticity of carcinogenesis we observed with either dietary or pharmacological E2 exposure. As the MMTV promoter is not directly responsive to E2, phenotypic and carcinogenic modulation by E2 was not expected. The mechanism behind this interaction is being explored and possible explanations include altered mammary gland structure (morphogenesis), signaling or other gene or expression changes. We have demonstrated greater phenotypic and histological variability (23,24; manuscript in preparation) than has been reported by others (40). These findings suggest that factors like E2 and phytoestrogens, in addition to the inserted erbB-2 transgene, may influence patterns of mammary gland and tumor development.

We believe that the underlying hormonal milieu, particularly during the early reproductive period, may be critical to mammary tumor development from benign mammary epithelium. Others are also exploring the long-term effect on mammary carcinogenesis of hormonal exposure of fetal or neonatal organisms. Interactions (either direct or indirect) between hormonal factors and the transgene will be further studied using our derived tumors and cell lines. Lines derived from the E2-casein group demonstrated the shortest doubling time, highest percentage in S phase by flow cytometry and greatest number of colonies in soft agar. Persistence of an aggressive phenotype in tumors and derived lines suggests a memory effect for E2 of benign glandular epithelium. A similar memory effect is observed in humans exposed to exogenous E2, tamoxifen or phytoestrogens, although the mechanisms of this effect are unknown.

In summary, we have studied the MMTV-wt-erbB-2 transgenic mouse model and demonstrated in vivo and in vitro modulation of this widely used model by E2 and phytoestrogen. We observe heterogeneous genomic and phenotypic characteristics in cell lines and mammary tumors, suggesting plasticity of the model system by the hormonal milieu. An aggressive phenotype in E2-treated mouse mammary gland tumors and derived cell lines was noted, consistent with a memory effect for E2 of mammary epithelial cells. In general, tumors in soy-fed mice occurred with a longer latency, displayed a greater number of chromosomal gains and losses as measured by CGH and were less aggressive in vitro. A high incidence of chromosome 4 loss was identified in tumors of the placebo/casein group and nearly half of the derived cell lines in this study. While the orthologous genes lost on chromosome 4 are not currently associated with known tumor suppressor genes in human breast cancer, this non-random finding may indicate genes on chromosome 4 that are interactive or synergistic to erbB-2.

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