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Aryl Hydrocarbon Hydroxylase Activity in Mouse, Rat, and Human Mammary Tumors[1]

Michelle E. Mason and Allan B. Okey[2]

Division of Clinical Pharmacology, Department of Paediatrics, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario Canada M5G 1X8

ABSTRACT

Aryl hydrocarbon hydroxylase (AHH) activity was measured in microsomes from chemically induced and spontaneous mammary tumors of mice and rats and in 213 human breast tumors. Basal enzyme activities [pmol 3-hydroxybenzo(*a*)pyrene per mg protein per min] ranged from 0.05 to 0.5 for rat, 0.05 to 10 for mouse, and 0 to 40 for human tumors. For comparison, mean liver AHH activities were 13 in untreated rats and 100 in untreated mice. Thus, some human breast tumors had AHH activity exceeding that in rat liver. Injection of 80 mg β-naphthoflavone per kg into tumor-bearing C3H/HeJ mice or Sprague-Dawley rats increased AHH activity to 10- to 70-fold over basal levels; there was no significant AHH induction in tumors from genetically "nonresponsive" DBA/2J or RF/J mice treated with β-naphthoflavone. α-Naphthoflavone in the incubation flask inhibited AHH activity in some human breast tumors and stimulated activity in others, probably reflecting the presence of multiple forms of cytochrome(s) P-450 in the human tumor population. AHH activity in human tumors was not correlated with their estrogen receptor content. Since several drugs used in cancer treatment are substrates for polysubstrate monoxygenases, high levels of AHH activity in some human tumors may play a role in their response to chemotherapy.

INTRODUCTION

Enzymes in various neoplastic tissues have been studied extensively (6, 16, 18). The purposes of such studies generally are 2-fold: (*a*) to determine if there are any consistent changes in enzyme patterns which might be markers of neoplastic transformation or might suggest the mechanisms of transformation; and (*b*) to determine if there are any biochemical differences between normal and neoplastic cells which can be exploited in therapy.

Polysubstrate monooxygenases such as AHH[3] have an important but complex role in neoplasia in experimental animals and probably in humans. These enzyme systems can introduce polar groups into hydrophobic chemicals such as polycyclic aromatic hydrocarbons, thus permitting them to be conjugated and excreted in an overall process of "detoxication"; however, in the process of metabolism, "reactive intermediates" may be generated which can act as proximate or ultimate carcinogens (9).

In addition to polycyclic aromatic hydrocarbons, polysubstrate monooxygenases also metabolize a wide variety of drugs and environmental chemicals (15), including several chemotherapeutic agents used in cancer treatment. Some chemotherapeutic agents appear to be tumoricidal only after "metabolic activation" (13) while the effectiveness of others may be impaired by rapid metabolism and excretion.

Since local metabolism within the tumor itself may play an important role both in metabolic activation and detoxication processes, we measured BP metabolism by AHH as a sensitive and convenient index of monooxygenase activity in 213 human breast tumors. AHH activity also was studied in several types of rodent mammary tumor in order to achieve a more comprehensive understanding of the nature and regulation of monooxygenases in neoplastic breast tissue.

MATERIALS AND METHODS

Chemicals. BP, NADPH, and NADH were purchased from Sigma Chemical Co. (St. Louis, Mo.), BNF and ANF were purchased from Aldrich Chemical Co. (Milwaukee, Wis.), and 3-hydroxybenzo(*a*)pyrene, produced by the IIT Research Institute (Chicago, Ill.), was a generous gift from Dr. Donald R. Mattison, NIH.

Animals and Tumor Procurement. Mammary tumors were induced in female C3H/HeJ, DBA/2J, and RF/J mice (The Jackson Laboratory, Bar Harbor, Maine) by daily i.p. injections of 0.5 mg BP or DMBA for 21 days starting at 6 weeks of age. Tumors first appeared about 6 months of age and were removed for study up until 1 year of age. Mammary tumors in C3H/HeJ were spontaneous tumors appearing in a colony of breeding females.

Mammary tumors in female Sprague-Dawley rats (Holtzman Co., Madison, Wis.) were induced by administration of a single p.o. dose of 15 mg DMBA given at 50 days of age. Tumors developed in 100% of the rats within 2 to 4 months after treatment; many rats had mammary tumors at multiple sites. Rat and mouse tumors were removed for study while they were firm and nonnecrotic; generally mouse tumors weighed 1 to 2 g at removal and rat tumors weighed 2 to 4 g.

Human breast tumors were received from the Ontario Cancer Foundation-Windsor Clinic, primarily to be assayed for estrogen receptor content in our laboratory; they represent 213 consecutive primary mastectomy specimens and were not selected from any particular stage or histopathological category.

Tumor Processing. Mouse and rat tumors were removed, trimmed of any necrotic portions, rinsed in iced TDE buffer, and frozen at −70° until the day of AHH assay. Human tumor specimens were trimmed of fat and any necrotic zones and then frozen in liquid nitrogen until the day of estrogen receptor

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; BP, benzo(*a*)pyrene; BNF, β-naphthoflavone (5,6-benzoflavone); ANF, α-naphthoflavone (7,8-benzoflavone); DMBA, 7,12-dimethylbenz(*a*)anthracene; TDE buffer, 10 mM Tris; 1 mM dithiothreitol; and 1.5 mM EDTA, pH 7.4.

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assay, at which time microsomes also were prepared from the tumor.

All tumor specimens were homogenized in TDE buffer (3 ml/g tissue) using a Polytron PT-10 homogenizer (Brinkmann Instruments, Inc., Rexdale, Ontario, Canada). TDE buffer was used for all tumors because it was the buffer required for estrogen receptor assays on human breast tumors. Tumor homogenates were centrifuged at 10,000 × *g* for 15 min, and the resulting supernatant then was centrifuged at 105,000 × *g* for 1 hr. All procedures were performed at 0–4° unless otherwise indicated. The supernatant from the 105,000 × *g* step constitutes the cytosolic fraction used for estrogen receptor determinations, whereas the pellet represents the microsomal fraction used for AHH assays. Microsomal pellets were resuspended in glycerol-phosphate buffer. Microsomes which were not assayed immediately were frozen at −70° for periods up to 2 months without apparent loss of AHH activity.

AHH Assays. AHH activity in tumor microsomes was measured essentially by the method of Nebert and Gelboin (10). The reaction was carried out at 37° using 100 μl of tumor microsomal suspension (3 to 10 mg protein/ml) in a final incubation volume of 1.05 ml. The complete reaction mixture contained: 0.25 ml of 0.2 M Tris (pH 7.5); 0.03 ml of 0.1 M $MgCl_2$; 0.62 ml of distilled water; 0.3 mg of NADPH; 0.3 mg of NADH; and 0.7 mg of bovine serum albumin. The reaction was initiated by addition of 0.1 μmol BP in 50 μl methanol to each reaction flask. In some experiments, the *in vitro* inhibitor of cytochrome P_1-450, ANF, was added in methanol to give a final concentration of 1 μM in the flask. The reaction was terminated and the products were extracted as described (10). AHH activity is expressed as pmol product formed per mg protein per min; 3-hydroxybenzo(*a*)pyrene, extracted under conditions identical with those of samples, was used as the standard. Activity was considered to be detectable when the sample fluorescence intensity was double that of the blank; this corresponds to 0.01 pmol/mg protein/min. Reactions catalyzed by tumor microsomes were linear at incubation times up to 60 min and at microsomal protein contents between 0.3 and 1.5 mg/flask. In the standard assay, tumor microsomes were incubated for 60 min and liver microsomes were incubated for 10 min.

AHH Induction by BNF Treatment *in Vivo*. Tumors in mice and rats were tested to determine if their AHH activities could be increased by exposure to a "methylcholanthrene-type" inducing chemical, BNF. BNF was injected i.p. at a dose of 80 mg/kg body weight for 2 consecutive days prior to removal of the tumor for assay. In some experiments, a portion of the tumor was removed by biopsy before BNF treatment, and then the animal was injected with BNF and the remaining portion of the tumor was reassayed for AHH activity after 2 days of BNF injection. Surgical procedures *per se* had no effect on AHH activity in the portion of the tumor remaining after biopsy (data not shown).

RESULTS

Enzyme Characteristics. BP metabolism by tumor microsomes required NADPH but not NADH or $MgCl_2$ (Chart 1*A*). Incubation at 22° or 0° reduced AHH activity to less than 10% of that observed at 37°; preheating microsomes to 60° or 100° nearly abolished activity. The optimal pH range was fairly broad, with maximal activity at approximately pH 7.5 (Chart 1*B*). Similar cofactor requirements, temperature dependence, and pH optima were obtained regardless of whether microsomes were from mouse, rat, or human mammary tumors.

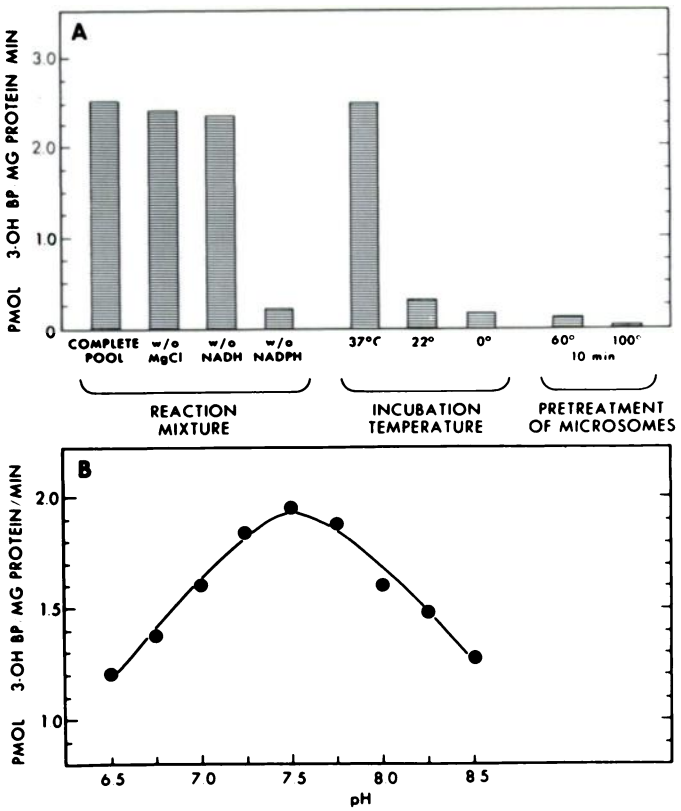

Chart 1. Optimal conditions for AHH activity in tumor microsomes. In *A*, microsomes from a DMBA-induced tumor (Sprague-Dawley rat) were prepared as described in "Materials and Methods" and then incubated under various conditions as indicated in the chart. The standard incubation time was 60 min at 37°. Each flask contained 0.7 mg of microsomal protein. In *B*, microsomes from a spontaneously occurring tumor (C3H/HeJ mouse) were incubated with a complete reaction mixture for 60 min at 37° in Tris buffer adjusted to the indicated pH values. Each flask contained 1.6 mg of microsomal protein.

To meet the requirements for estrogen receptor assays on human tumors, all tumors were homogenized in TDE buffer rather than the phosphate buffer system usually used for microsome preparation (10). AHH activity was compared in portions of DMBA-induced rat tumor homogenized in either TDE buffer or phosphate buffer. AHH activity was slightly higher when tumors were homogenized in TDE buffer, but the difference from samples homogenized in phosphate buffer was not statistically significant. Microsomal protein yields were slightly lower using TDE as the homogenizing buffer compared with homogenization in phosphate buffer (data not shown).

AHH Activity in Mouse Mammary Tumors. Basal AHH activity was detectable in mammary tumors from untreated (no BNF injections) C3H/HeJ, DBA/2J, and RF/J mice (Chart 2). Injection of BNF caused a marked induction of AHH in tumors from the genetically "responsive" C3H/HeJ strain, typically producing greater than 10-fold increases over basal activity measured in biopsy samples from the same tumor before BNF treatment. BNF injection into genetically "nonresponsive" DBA/2J and RF/J mice produced marginal increases in tumor AHH, averaging only 1.5-fold higher than basal (Chart 2).

Addition of 1 μM ANF to the reaction flasks consistently

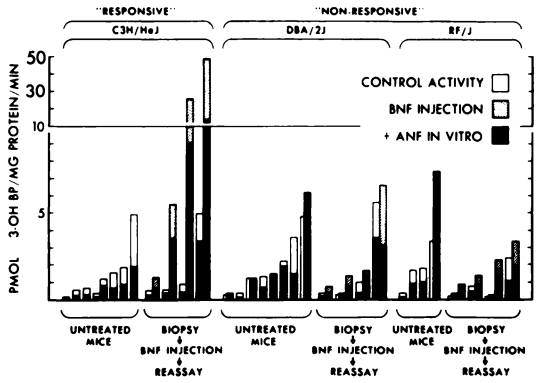

Chart 2. AHH activity in individual mouse mammary tumors. Tumors were obtained as described in "Materials and Methods." □, control (basal) AHH activity in tumors or tumor biopsies removed before BNF injection; ▨, AHH activity in tumors from mice injected with 80 mg BNF per kg body weight 48 and 24 hr before removal of the remaining tumor. BNF-induced values are adjacent to basal activity measured in a biopsy taken from the same tumor before BNF injection. ■, AHH activity measured in the presence of 1 μM ANF in the reaction flask. Note that basal activity in tumors from some untreated animals is higher in the presence of ANF than without ANF; the *solid columns* in these cases are slightly offset from the corresponding control values.

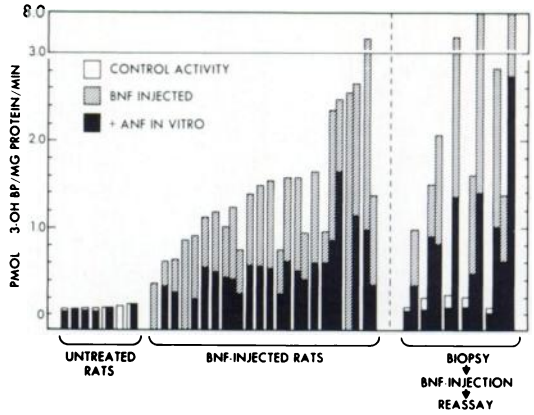

Chart 3. AHH activity in individual rat mammary tumors. Tumors were obtained as described in "Materials and Methods." □, control (basal) AHH activity in tumors or tumor biopsies removed before BNF injection; ▨, AHH activity in tumors from rats injected with 80 mg BNF per kg body weight 48 and 24 hr before removal of the remaining tumor. Clustered adjacent *columns* represent multiple tumors removed from the same rat. ■, AHH activity measured in the presence of 1 μM ANF in the reaction flask. Absence of a *solid portion* in the *column* indicates that ANF effect was not tested in that particular tumor.

inhibited basal AHH activity in tumors from C3H/HeJ mice but occasionally caused apparent stimulation of basal activity in tumors from DBA/2J or RF/J mice. When microsomes were prepared from BNF-treated mice of any strain, ANF *in vitro* inhibited AHH activity by at least 50%.

Tumor AHH activities were much lower than activities in liver microsomes from the same animals. Basal liver AHH activity in tumor-bearing C3H/HeJ mice was 102 ± 44 pmol/mg/min, and this increased to 1041 ± 188 after BNF injection.

AHH Activity in Rat Mammary Tumors. AHH activity in microsomes from DMBA-induced rat mammary tumors (Chart 3) was lower than that observed in mouse mammary tumors (Chart 2). Although basal activities were extremely low, BNF injection caused dramatic induction of tumor AHH activity, averaging 70-fold increases over basal values (Chart 3; Table 1). Basal and BNF-induced AHH activities in tumors were of similar magnitude to those in nontumorous mammary tissue from the same animals (Table 1).

AHH inhibition by ANF *in vitro* was greater against tumor and mammary microsomes from BNF-treated rats than against microsomes from untreated controls.

Several rats had tumors at multiple sites; thus, we examined the data to determine if multiple tumors within one animal (subject to the same host influences) were less variable in AHH activity than tumors occurring in different animals. By ratio of variances (*F* test), tumors within individual rats were as variable in AHH activity as the tumor population from all rats (h_0, $p < 0.05$). Tumors also were no more variable in their AHH activity than livers or nontumorous mammary tissues of the tumor-bearing rats.

AHH Activity in Human Breast Tumors. The 213 human breast tumors assayed (Chart 4*A*) displayed a wide range of AHH activities. Activity was nondetectable in 15% of the tumors and was near detection limits (0.01 pmol/mg/min) in another 40%. However, about 20% of the tumors had activities greater than 0.5 units and a few tumors had activity exceeding that in uninduced rat liver. The tumor population was heterogeneous in regard to the effects of ANF *in vitro*. In tumors with detectable AHH, ANF inhibited activity in 50%, had no effect in 30%, and stimulated activity in 20%.

Table 1

Summary of AHH activity in rat mammary tumors and in nontumorous mammary tissues and livers from the same animals

Tissue	AHH activity (pmol/mg/min) Control	*n*	+1 μM ANF *in vitro*
Mammary tumors			
Basal	0.05 ± 0.01[a]	16[b]	0.025 ± 0.01
BNF-induced[c]	3.35 ± 1.5	36[b]	0.99 ± 0.33
Nontumorous mammary tissue			
Basal	0.02 ± 0.01	8	0.01 ± 0.01
BNF-induced	2.5 ± 0.32	24	0.48 ± 0.005
Liver			
Basal	13.1 ± 3.8	8	6.5 ± 1.6
BNF-induced	510 ± 22	24	290 ± 19

[a] Mean ± S.E.
[b] Multiple tumors often were assayed from one rat, thus *n* is greater for tumors than for mammary tissues or liver.
[c] Activity induced by injection of 80 mg BNF per kg body weight 48 and 24 hr before tumor removal.

Log-transformed frequency distributions of AHH activity in mouse and rat tumors were unimodal and normally distributed about the mean (data not shown). The human breast tumor frequency distribution was highly skewed to the left (low activity) and, rather than being unimodal, contained a second cluster at higher activities (Chart 4*B*).

AHH activity was not correlated with estrogen receptor levels in the same tumors ($n = 213$; $r = -0.03$).

DISCUSSION

General characteristics of the AHH enzyme system in rodent mammary tumors are similar to those observed in normal mammary tissue of the same animals. This is especially true in regard to induction of activity by BNF injection. AHH activity is highly induced in tumors from genetically responsive animals such as C3H/HeJ mice and Sprague-Dawley rats; the degree of induction is similar to that observed in nonmalignant mam-

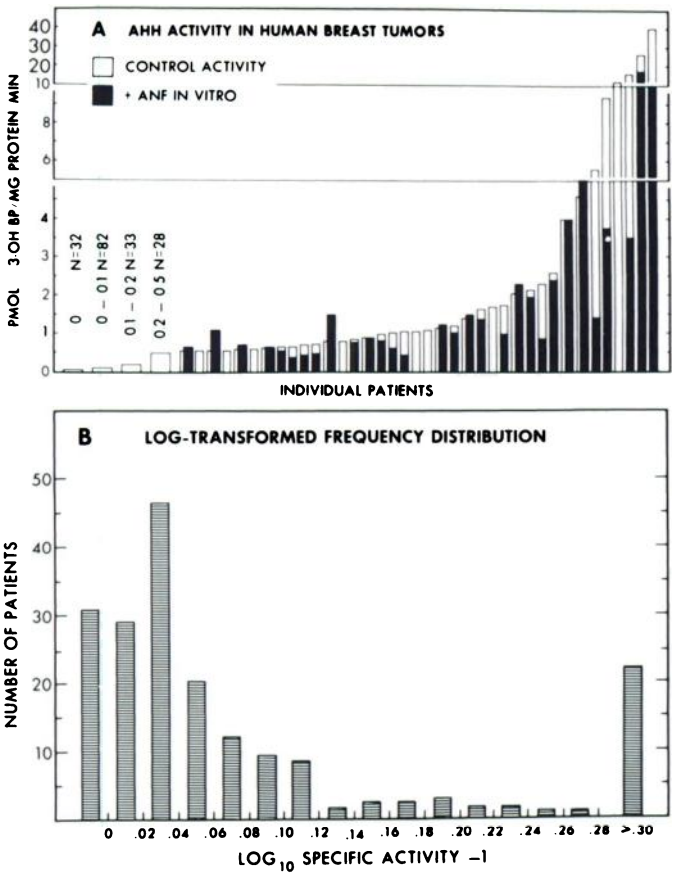

Chart 4. AHH activity in human breast tumors. *A*, activity in individual tumors. Tumors with low activity have been grouped, and the number of tumors within each interval is indicated. □, control values for individual tumors; ■, AHH activity measured in the presence of 1 μM ANF in the reaction flask. Note that activity in some tumors is higher in the presence of ANF than without ANF; the *solid columns* in these cases are slightly offset from the corresponding control (no competitor) values. Absence of a *solid portion* in the *column* indicates that ANF effect was not tested in that particular tumor. *B*, log-transformed frequency distribution. The $\log_{10}$ of AHH activity was determined for each breast tumor and plotted in a frequency distribution.

mary tissues from the same strains (1, 3, 4). BNF injection has little effect on AHH activity in mammary tumors from the genetically nonresponsive DBA/2J and RF/J strains, in accord with failure of normal tissues in these animals to exhibit significant AHH induction when exposed to methylcholanthrene-type inducing chemicals (9, 15). Thus, the process of malignant transformation in mammary cells does not appear to cause frequent aberrations in genetic regulation of AHH induction.

Previous studies of AHH activity in tumors have concentrated on various hepatomas (12, 14, 17); generally, AHH activity in hepatomas is less than one-half of that observed in normal liver cells from the same animals whereas the activity in mammary tumors is somewhat higher than that in normal nonlactating mammary tissue.

As might be expected, AHH activity was more variable in human breast tumors than in tumors from laboratory animals. Most human tumors had very low AHH activity, but some had activities exceeding that in liver microsomes from untreated rats. Human tumors also were heterogeneous in their response to ANF *in vitro*; ANF inhibited AHH activity in some tumors and stimulated activity in others. ANF is thought to preferentially inhibit metabolism associated with enzymes induced by methylcholanthrene-type chemicals (7). At 100 μM concentrations, ANF previously has been shown to stimulate BP metabolism by some human liver homogenates, but there was considerable individual variation in the response to ANF, probably due to the presence of multiple forms of cytochrome(s) P-450 in human tissues (7). ANF may stimulate metabolism of BP by certain forms of P-450 and inhibit BP metabolism by other forms. The heterogeneity of response to ANF observed in human breast tumors also may reflect the presence of multiple species of P-450 in different tumors.

The genetic regulation of AHH induction in humans is complex and poorly understood. In inbred strains of mice, AHH induction is regulated by the *Ah* complex which involves a small number of loci; although there is considerable evidence for heritable variation in AHH inducibility in humans, it is not yet clear whether induction is controlled by a single genetic locus or inheritance is polygenic (reviewed in Ref. 15). The skewed frequency distribution of AHH activities in human breast tumors in this study does not allow interpretation of the genetic basis for control of AHH in the tumors. It is possible that human tumors having the highest AHH activities may be from highly "inducible" individuals who have been exposed to an inducing chemical. Smoking records for the population and other histories of drug or chemical exposure were not sufficiently complete to allow determination of the relationship between AHH activity and possible intake of chemical inducers.

The enzyme activity (BP hydroxylation) assessed in this study was chosen because it can be measured with a highly sensitive assay. BP is metabolized by more than one form of cytochrome P-450 (9), and the various forms of P-450 detected by this assay may have significant effects on metabolism of drugs commonly used in cancer chemotherapy. For example, the form of cytochrome P-450 induced by BNF decreases cyclophosphamide activation by rat liver (5) and increases the conversion of azoprocarbazine to the methylazoxy derivative (19). Cytochrome P-450 induced by phenobarbital treatment increases the conversion of cyclophosphamide into alkylating metabolites (13) and increases the metabolism of procarbazine into azoprocarbazine (2). It also appears that cytochrome P-450-mediated monooxygenases are involved in the activation of mitomycin C (8).

Most studies on the metabolic activation or inactivation of drugs used in cancer chemotherapy have focused on enzyme systems located in liver, lung, and kidney. As we have demonstrated in this report, some human breast tumors contain significant levels of monooxygenase activity. It is possible that local metabolism of chemotherapeutic agents within the tumor itself may significantly alter the response of that tumor to the drug. Follow-up studies currently are in progress to determine whether there is any association between levels of AHH activity in primary human breast tumors and subsequent response of recurrent or metastatic lesions to chemotherapy.

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