Is aryl hydrocarbon hydroxylase activity a new prognostic indicator for breast cancer?

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Summary Aryl hydrocarbon hydroxylase (AHH) activity was measured in the breast tumours of 153 primary and 17 recurrent cancer patients, and in 18 patients with benign breast tumour. All operations were carried out in 1983–84. The cytosolic fraction was collected for steroid receptor determination, and microsomes were separated for AHH assay from the same tissue samples. The AHH distribution was wide and highly skewed in all groups. About 10% of the samples showed activities below detection limit. The medians and ranges for primary cancers were 34 (<5–2683), for recurrent cancers 40 (20–239) and for benign tumours 11 (<5–37) $fmol\,min^{-1}\,mg^{-1}$ protein. After logarithmic transformation, the mean AHH activities of cancer samples differed significantly from those of benign tumours. The logarithm of AHH activity (log AHH) correlates positively with axillary lymph node status, and negatively with steroid receptor levels. The development of the disease and the survival of the patients were followed for 4 years. The survival and the disease-free interval of the cancer patients who had low AHH activity was significantly higher than that of the high AHH group. The multivariate analysis with Cox's proportional hazad model showed primary tumour size, progesterone receptor concentration, nodal status and log AHH to be the most important independent prognostic factors for survival, while the occurrence of metastases, log AHH and tumour size were the equivalent factors for the disease-free interval in primary breast cancers. We conclude that AHH activity may reflect the overall malignant potential of breast cancer tissue.

The cytochrome P450-dependent monooxygenases play a key role in the metabolism of a wide variety of xenobiotics, such as drugs, environmental pollutants, carcinogens and also steroids, including several chemotherapeutic agents used in cancer treatment. Cytochrome P450 is found in animals and in humans as multiple isoenzymes (Nebert & Conzalez, 1987), the relative amounts and activities of which may largely govern the balance between bioactivation and detoxification of a particular compound (Pelkonen & Nebert, 1982). Aryl hydrocarbon hydroxylase (AHH), a cytochrome P450 activity induced by polycyclic aromatic hydrocarbons (PAH), is considered the most important enzyme in the metabolic activation of several compounds, especially PAH, into ultimate carcinogenic forms (Pelkonen & Nebert, 1982). The induction of AHH is genetically controlled by Ah-receptors (Nebert & Gonzalez, 1987). In animal models, levels of AHH activity are linked to susceptibility to chemical carcinogen induced sarcomas, skin carcinomas, lung carcinomas, and lymphomas and leukaemias (Kouri *et al.*, 1982). Many efforts have been made to determine a relationship between AHH activity or inducibility and susceptibility to lung cancer or other forms of cancer also in human populations, but the results vary and are mostly contradictory (Kouri *et al.*, 1982; Kärki *et al.*, 1987).

The actual cause of breast cancer is unknown. Probably it is a multietiological as well as a multiform disease. Several risk factors have been found, including genetic, hormonal, nutritional, physical and morphological factors (Lynch *et al.*, 1984; Thomas, 1984; Mettlin, 1984). The development of breast cancer, the occurrence of metastases and the survival of patients vary from case to case. The role of chemical carcinogenesis in human breast cancer is unclear. However, in mice and rats, mammary tumours can be induced by treatment with polycyclic aromatic hydrocarbons, such as benzo(a)pyrene (BP) and 7,12-dimethylbenz(a)anthracene (Furr & Jordan, 1984).

In the present study, we measured AHH activities in benign and malign human breast tumours and compared the results to the survival of patients and to the development of the disease in 4-year follow-up time.

Materials and methods

Patients and samples

Breast samples were received from a total of 188 patients, 18 with benign breast tumour, 153 with primary breast carcinoma, and 17 with recurrent or new breast cancer at 1–23 years after the first mastectomy. The patients were operated in 1983–84 in the University Hospital of Tampere or in surrounding hospitals. The samples (0.5–2 g) were taken primarily for a routine assay of oestrogen and progesterone receptor content in the tumour tissue. They were placed in small plastic bags, immediately frozen in liquid nitrogen and brought to laboratory, where they were stored at $-70°C$ until the day of steroid receptor assay, at which time the microsomes were also prepared.

Data age, tumour size, metastatic axillary lymph nodes and other metastases according to TNM classification (Beahrs, 1984) of patients were recorded (Table I). All benign tumour patients had fibrocystic disease. In the cancer patient group, infiltrating ductal cancers accounted for 89% of the primary and for 71% of recurrent cases, the remaining cases being distributed among lobular, mucinous and medullary histologic types.

Preparation of tumour cytosole and microsomes

The frozen samples were pulverised with a Braun dismembrator, suspended in cold 10 mM Tris-HCl buffer, pH 7.5, containing 10% glycerol, and centrifuged at 105,000 g for 1 h at $+4°C$. The supernatants consisting of the cytosolic fraction were used for steroid receptor determinations, whereas the pellets were resuspended with a Potter-Elvehjelm glass-teflon homogeniser in 0.1 M K-phosphate buffer, pH 7.4, and recentrifuged at 13,000 g for 10 min at $+4°C$. The supernatants contained the microsomal fractions and were used for the AHH assay.

Table I Preoperative characteristics of patients with benign breast tumour (Group A), primary breast cancer (Group B) and recurrent breast cancer for second mastectomy (Group C)

	Group A	Group B	Group C
Number of patients	18	153	17
Age:			
median, years	45	59	61
range	22–68	27–83	41–72
TNM classification:			
T1, No.		57	5
T2		61	6
T3		12	2
T4		20	3
unknown		3	1
N0		75	11
N1		64	4
N2		9	1
N3		2	0
unknown		3	1
M0		149	16
M1		4	0
unknown			1

Table II Steroid receptors and anti-estrogen therapy of patients with primary breast cancer (Group B) and recurrent breast cancer for second mastectomy (Group C)

	Group B	Group C
Number of patients	153	17
Steroid receptor status:		
ER – PR –, No.	35	5
ER – PR +	16	4
ER + PR –	16	2
ER + PR +	85	6
unknown	1	
Oestrogen receptor concentration:		
median	17	<2
range	<2–655	<2–213
Progesterone receptor concentration:		
median	36	<3
range	<3–2632	<3–897
Anti-oestrogen therapy:		
yes	74	11
no	76	6
unknown	3	

Steroid receptor assays

Both oestrogen (ER) and progesterone receptors (PR) of breast cancer tissues were determined by the dextran charcoal method (Koreman & Dukes, 1970). The oestrogen receptor concentrations were considered positive (ER +) when they contained 4 fmol mg^{-1} protein or thatover, and negative (ER –) when they were under that level. Progesterone receptor concentrations were considered positive (PR +) when they contained at least 7 fmol mg^{-1} protein and negative (PR –) when they contained less.

AHH assay

AHH activity in tumour microsomes was measured by the fluorometric method (Nebert & Gelboin, 1968). The reaction was carried out at 37°C for 60 min in dark using 900 μl of tumour microsomal suspension (0.2–4 mg protein ml^{-1}) in a final incubation volume of 1 ml. An aliquot of every sample suspension was boiled for 10 min and assayed with samples for individual blank correction. AHH activity is expressed as fmol product (equivalent to 3OH-BP) formed per mg protein per min. The activity was considered to be detectable when the difference of the active and the boiled sample fluorescence intensity was twice as high as the variation in the duplicate determination. This corresponds to 5 fmol mg^{-1} protein min^{-1}. The protein concentration was determined according to Peterson (1977) using bovine serum albumin as a standard.

Treatment and follow-up of patients

All cancer patients were treated with mastectomy and, by only a few exceptions, with evacuation of the same side axillary lymph nodes. After the operation, the patients were sent to an oncological unit, where radiation therapy was given to all patients whose tumour size was T1N1M0 or larger. Anti-oestrogens (tamoxifen) or cytotoxic drugs were administered individually according to steroid receptor and metastatic status (Table II). According to the routine treatment schedule used in Finland, the patients thereafter visited the oncological out-patient clinic every 3–6 months. The relapses and deaths of patients were recorded, and the status of patients exactly 4 years after the operation was used in survival evaluation.

Statistics

Data processing was carried out on an Olivetti M280 computer, and the appropriate statistics were obtained using SYSTAT package (Wilkinson, 1986), BMDP1L and BMDP2L programme (Dixon, 1985). The differences between the means were tested by the two-sample t-test or by one-way analysis of variance followed by the Newman-Keuls test. Also, Pearson correlation analysis and χ^2 test were performed. The survival and disease-free interval curves were constructed according to the Kaplan-Meier method. The statistical differences between the curves were calculated using log-rank test according to Mantel and Cox. The relative importance of prognostic factors was assessed with Cox's proportional hazard model. The proportionality assumptions were tested using a time-dependent covariate.

Results

### Overall characteristics and survival experience of patients

The median ages increased in our patient groups from benign-tumour Group A to primary-cancer Group B and to recurrent or second-cancer Group C (Table I), which followed the development time of these diseases. The proportion of small (T1) and local (N0, M0) tumours was a little less in the primary carcinoma group than in the recurrent carcinoma group, but the difference was not statistically significant, because of the small size of Group C. The steroid receptor status and receptor concentrations of breast cancer patients (Group B and C) are given in Table II. In Group C, there were less steroid receptor-positive (ER + PR +) tumours than in Group B ($\chi^2 = 6.93$, $P < 0.01$), and the medians and ranges of concentrations were lower.

At the end of the 4-year follow-up time after the surgical operation, all patients with benign breast tumour ($n = 18$) were disease-free. Of the primary cancer patients ($n = 153$), 83 were disease-free, but 70 patients had relapsed and developed metastases during the follow-up time, and 38 patients of them died. Of the recurrent cancer group ($n = 17$), nine patients were alive and eight dead after the 4-year follow-up from the second mastectomy.

AHH activities

The distributions of the AHH activities were wide and highly skewed in all groups (Figure 1). The major part of AHH activities in human breast tumour samples were very low; about 10% were below the detection limit of the assay. However, some malignant tumours showed over ten times higher AHH activities compared to those of the benign tumours.
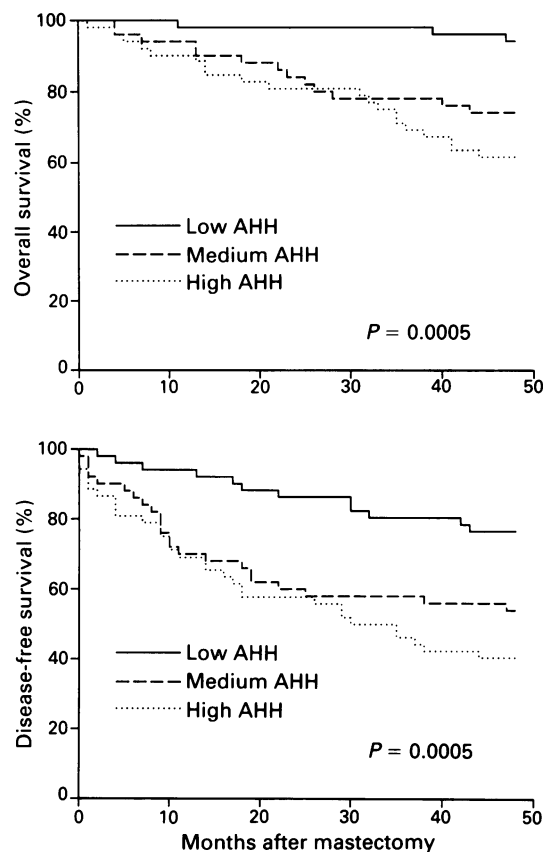
Table III shows the medians and ranges of the AHH activities of various patient groups and subgroups. Logarith-
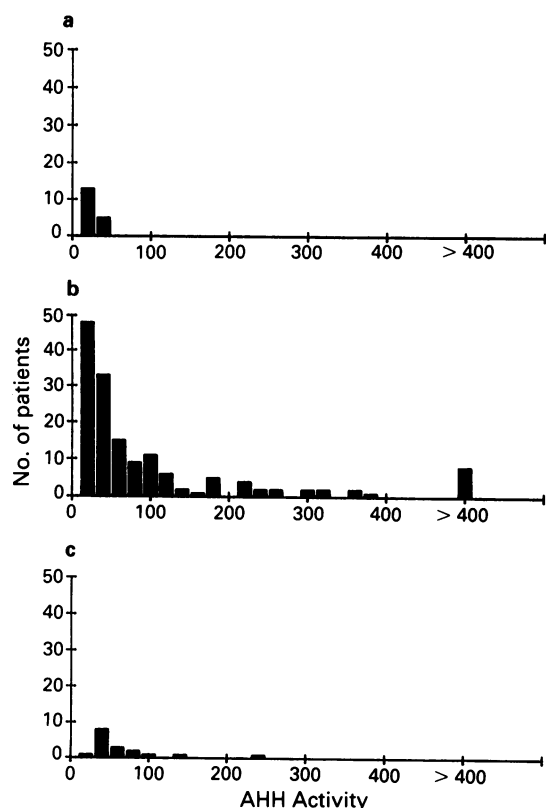
Figure 1 The frequence distribution of AHH activities in patients with benign breast tumour a, primary breast cancer b, and recurrent breast cancer c.

Figure 2 Overall survival curves (upper) and disease-free survival curves (lower) for 153 patients with primary breast cancer by AHH activity in cancer tissue. The groups are: LOW (AHH activity <24 fmol min^{-1} mg^{-1} protein, $n = 51$), MEDIUM (AHH activity 24–75 fmol min^{-1} mg^{-1}, $n = 50$) and HIGH (AHH activity >75 fmol min^{-1} mg^{-1}, $n = 52$). The significance of difference in survival is given according to a Mantel-Cox test.

mically transformed AHH activities were normally distributed about the mean, which allows the analysis of variance. The mean of log AHH in samples from benign tumours differed significantly from those of cancer samples. The log AHH activities of primary cancer patients who later on were disease-free are significantly different from the values of those who died. Differences between recurrent cases and disease-free or dead patients were not statistically significant in Group B. Among the recurrent cancer patients (Group C) there was no prognostic difference between log AHHs of living and deceased patients.

The primary cancer patients ($n = 153$) were divided into three groups according to AHH activity as follows: the low AHH group contained cases in which the AHH activity was less than 24 fmol min^{-1} mg^{-1} ($n = 51$), the medium AHH group showed activities between 24 and 75 fmol min^{-1} mg^{-1} ($n = 50$), and the high AHH group showed activities above 75 fmol min^{-1} mg^{-1} ($n = 52$). A clear prognostic difference both in overall and in disease-free survival could be identified between the groups (Figure 2). The survivals were poorer when AHH activity was higher. The low-AHH group differed

from the others, but the differences between medium and high AHH groups were not statistically significant.

Relation to steroid receptor level and tumour stage

The AHH activities of primary breast cancer patients did not correlate with the patient's age, the concentration of ER or PR in tumour tissue, or T, N or M stage. Because no AHH or ER or PR are normally distributed, their logarithms are also included in the correlation analysis. Then the logarithm of AHH activity correlated positively with increasing lymph nodes ($r = 0.238$, $P < 0.01$), and negatively with logarithm of oestrogen receptor concentration ($r = -0.300$, $P < 0.001$) and the logarithm of progesterone receptor concentration ($r = -0.220$, $P < 0.01$). T, N and M-classes correlated with each other.

Table III   AHH activities in human breast tumours

Group	Disease of patient	N	AHH (fmol mg^{-1} min^{-1}) Median	Range	log AHH Mean	s.d.	Different from groups[a]
A	Benign tumours:						
1- all		18	11	$<5-37$	0.833	0.590	2,3,4,5,6,7,8
B	Primary cancers:						
2 -all		153	34	$<5-2683$	1.564	0.666	1,5
3 -disease free		86	27	$<5-488$	1.425	0.651	1,5
4 -recurrent, alive		31	56	$<5-614$	1.597	0.669	1
5 -recurrent, dead		36	82	$<5-2683$	1.869	0.608	1,2,3
C	Recurrent cancers:						
6 -all		17	40	$20-239$	1.696	0.262	1
7 -alive		9	39	$20-239$	1.707	0.324	1
8 -dead		8	43	$28-122$	1.686	0.193	1

[a]Differences between the log AHH of the groups were tested by variance analysis followed by Newman-Keuls test, $P < 0.05$.

Multivariate analysis

In order to find out the relative importance and independence of AHH activity as a prognostic factor, it was tested in Cox's proportional hazard model together with other factors whose prognostic significance was proposed. The variables in the model included initially AHH activity (fmol min^{-1} mg^{-1} protein) as well as its logarithm, the concentration of oestrogen and progesterone receptor (fmol mg^{-1}), axillary nodal status (coded: N0-1 = 0, N2-3 = 1), primary tumour size (coded: T1 = 1, T2 = 2, T3 = 3, T4 = 4), and distant metastases (coded: M0 = 0, M1 = 1). The logarithm of AHH activity proved a better covariate than untransformed AHH activity. The proportionality of hazards assumption was tested and found to be valid. Table IV summarises the results of stepwise selection procedures. The most important independent prognostic factor for overall survival in our primary breast cancer patients group appeared to be tumour size, followed by progesterone receptor concentration, nodal status and the logarithm of AHH activity. The independent prognostic factors for disease-free survival were the presence of metastases, the logarithm of AHH activity and tumour size. Nodal status and steroid receptors showed less independent impact on disease-free survival prognosis.

Anti-oestrogen therapy

After mastectomy, about half of the primary carcinoma patients were treated with anti-oestrogen (tamoxifen) (Table II). Most of these patients (65/74) had cancer which TNM class was over T1N0M0, and 33 of them died during the 4-year follow-up time regardless of the therapy. As in the whole cancer patient group, also in this subgroup significantly more deaths occurred in the high AHH group than in the low AHH group (χ^2 = 8.64, $P < 0.01$). However, on the basis of our results, it is not possible to separate the additional influence of the possibly increased anti-estrogen metabolism from other factors affecting the distribution of survivals in our patient material.

Discussion

The low activities and the wide variation of AHH activities in human breast tumours found in the present work were similar to those reported by Mason and Okey (1981). Large interindividual variations in AHH activities were also found in other human extrahepatic tissues, such as lung (Cohen et al., 1979; Sabadie et al., 1981; Roberts et al., 1986), lymphocytes (Kellerman et al., 1973) and placenta (Gurtoo et al., 1983). The wide distribution of AHH activities in tumour samples may be due to a different basal level, a different level of induction, a different response to inducers, or to a combination of these factors. A cause for the highest activities may be a genetic or a carcinogenesis-derived defect of the regulation mechanism, which may lead to induction without or regardless of inducer (Neber & Gonzalez, 1987).

Our main result, which is that high AHH activity in breast cancer tissue predicts a bad prognosis of the disease, is a new one. The AHH activity of benign tumours was significantly lower than that in carcinomas and cancers with good prognosis lower than that of bad prognosis (though there were many individual exceptions). This suggests unavoidably the possibility of AHH-mediated carcinogenic activation in the most aggressive tumours.

Both higher and lower AHH activities in tumours compared to normal tissue have been reported to occur in human and chemically induced animal cancers. Human uterine leiomyomas were reported to show higher cytochrome P450 activity than adjacent normal myometrium (Senler et al., 1985b). In rat mammary tumours, higher AHH activities were found than in normal nonlactating mammary tissue (Mason & Okey, 1981), whereas in most of the lung cancer patients, AHH activity was lower in the tumorous tissue than in the surrounding tissue (Sebadie et al., 1981). The discrepancies of results probably indicate the difficulties to control or to identify all the possible factors, as well as the enormous heterogeneity of the various forms of cancers.

Animal experiments have shown that susceptibility to several types of polycyclic aromatic hydrocarbon-induced tumours is strongly associated with the Ah receptor-mediated inducibility of AHH (Pelkonen & Nebert, 1982). AHH induction has also been confirmed in human tissues. It is induced in placenta by smoking (Pasanen et al., 1988b) and in cultured lymphocytes by treatment with chemicals such as bentz(a)antracene (Kouri et al., 1982; Kellerman et al., 1973). Ah receptors were found in human tissues, and the induction mechanism was considered to be the same as in mice and rats. It has also been suggested that genetic differences in AHH inducibility in humans might be important in determining susceptibility to some cancer forms (Kouri et al., 1982). But again, the results from different laboratories vary. Kouri et al. (1982) reported a positive correlation between high AHH inducibility in cultures of human lymphocytes and the occurrence of primary lung cancer, whereas Kärki et al. (1987) found no difference between the lung patients and the controls. Also, no association was found between AHH inducibility and occurrences of leukaemias or solid tumours in children (Levine et al., 1984).

Our results do not confirm AHH-mediated chemical carcinogenesis in breast cancer, though it does not disprove it, either. A considerable part of cancer samples and all benign tumours showed very low AHH activity in our study as well as in earlier ones (Mason & Okey, 1981). It is not probable that AHH activity in those cases should have any importance. The etiology of at least these tumours may be different and independent of AHH. Several human cells in culture have been shown to contain cytochrome P450 and exhibit AHH induction in response to treatment to various compounds. In a human breast cancer cell line, AHH activity was induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Different cell lines vary greatly with respect to the basal expression levels of cytochrome P450 and its inducibility by TCDD (Pasanen et al., 1988a). Some human breast tumour cell lines, though derived from carcinomas, were practically

Table IV Importance of independent prognostic factors on disease-free survival (A) and overall survival (B) of primary breast cancer patients (n = 153). Summary of stepwise results of Cox's proportional hazard model

Step no.	Factor (variable entered)	Improvement Chi-square	P-value	Global Chi-square	P-value
A. Overall survival:					
1	Tumour size	21.131	0.000	25.806	0.000
2	Procesterone receptors	17.744	0.000	30.612	0.000
3	Nodal status	9.479	0.002	56.652	0.000
4	Log AHH activity	7.058	0.008	62.939	0.000
B. Disease-free survival:					
1	Metastases	14.382	0.000	47.499	0.000
2	Log AHH activity	15.950	0.000	61.839	0.000
3	Tumour size	3.742	0.053	65.228	0.000

non-responsive to induction (Pasanen et al., 1988a).

The present knowledge about the association of chemical carcinogenesis to breast cancer mediated by AHH activity seems not to be enough to give an answer to why high AHH activity associates to a poor prognosis of breast cancer. A clarification of its causes and importance will need much further work. We do not know yet whether high AHH activity is the cause or a consequence of malignant transformation. Also, it may be an independent change, which, however, is concentrated in the most serious breast cancer cases.

The most important prognostic indicator for breast cancer have been found to be tumour size and the extent of axillary lymph node involvement (Carter et al., 1989). Lymph node status seems to serve as an indicator of a tumour's ability to spread. Distant metastases are the cause of deaths in most patients. In the present work, logarithm of AHH activity in carcinomas correlated positively with the occurrence of axillary lymph nodes. Tumour size, the presence of nodal and distant metastases correlated with each other. The three most important prognostic factors for disease-free intervals in our primary breast cancer patients were found to be metastases, log AHH and tumour size. For the overall survival, the importance of nodal status and progesterone receptors were also as a prognostic factors revealed, but log AHH still included in the independent prognostic factors.

Some negative correlation after logarithm transformation was found between AHH activity and steroid receptors. The presence of oestrogen and progesterone receptors in breast tumour increases the likelihood that the patient will respond to endocrine therapy, but higher levels of oestrogen receptors are associated with a greater probability of disease-free survival (Carter et al., 1989).

Because cytochrome P450 enzymes metabolise tamoxifen as well as other anti-estrogens (Robinson & Jordan, 1988), a high AHH activity may correlate with enzymes which inactivate the chemotherapeutic agents within the tumour itself, and may alter significantly the response of the tumour to the drug (Senler et al., 1985a). This could explain the poor prognosis of our high-AHH patients, who were treated with antiestrogens. Because our patient material was too heterogenous, the results did not give any clear confirmation to this interesting possibility. However, the increased metabolism of anti-oestrogens cannot explain all our results, and a better clarification of this problem will be the aim for our future studies.

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