[CANCER RESEARCH 59, 2825–2828, June 15, 1999]

Advances in Brief

CYP17 and Breast Cancer Risk: The Polymorphism in the 5′ Flanking Area of the Gene Does Not Influence Binding to Sp-1[1]

Vessela Nedelcheva Kristensen,[2] Ellen K. Haraldsen, Kristin B. Anderson, P. E. Lønning, Bjørn Erikstein, Rolf Kåresen, Odd S. Gabrielsen, and Anne-Lise Børresen-Dale

Department of Genetics [V. N. K., E. K. H., A-L. B-D.] and Department of Oncology [B. E.], Institute of Cancer Research, the Norwegian Radium Hospital, University Clinic, Montebello 0310, Oslo; Department of Biochemistry, University of Oslo, Oslo 0316 [K. B. A., O. S. G.]; Department of Oncology, Haukeland Hospital, 5021 Bergen [P. E. L.]; and Department of Oncology, Ullevål Hospital, Oslo 0407 [R. K.], Norway.

Abstract

The ability of a motif of the *CYP17* 5′ untranslated region, created by a polymorphic T to C substitution, to bind to the human transcription factor Sp-1 was investigated. No binding of any of the polymorphic alleles was observed in electromobility shift assay. No other sequence within +1 to +100 of each of the *CYP17* alleles formed complex with the Sp-1 or enhanced binding to the polymorphic CACC box. Genotyping of 510 breast cancer patients and 201 controls revealed no difference in genotype frequencies. Age at onset, tumor grade, lymph node status and distant metastases, stage, and estrogen and progesterone receptor status were not associated with the *CYP17* genotype.

Introduction

Cytochrome P450c17 is a key enzyme in the sex steroid synthesis (1). The enzyme catalyzes the 17α-hydroxylation of pregnenolone and progesterone. The same enzyme also catalyzes the conversion of C21 steroids to C17. Both activities are required for androgen and estrogen synthesis (1, 2). A role for estrogen in human breast carcinogenesis is supported by epidemiological risk factors, as well as by recent studies linking plasma estrogen levels to breast cancer risk. (3, 4; reviewed in Ref. 5). It has been hypothesized that the 17α-hydroxylase/17,20lyase activity may be at least one of the biochemical determinants of these phenomena. *CYP17*, the gene coding for this enzyme, maps to chromosome 10 and contains eight exons and seven introns (6). A polymorphism (a T-C substitution) in the 5′ UTR[3], at +27 relative to the start of transcription, has been described (7, 8). Feigelson *et al*. (8) demonstrated that the C (*A2*) allele was more frequent in postmenopausal patients with advanced breast cancer than in controls and suggested this variant to be associated with an increased risk of the disease. In a recent study, the variant *A2* allele was found to be associated with higher serum hormone levels in young healthy individuals (9). The substitution has several times been discussed to create a putative Sp-1 binding site (CCACT-CCACC), thus providing a mechanism for higher expression of the variant allele (8, 9). The constitutive transcription factor Sp-1 plays a role in the transcription of numerous cellular genes, including constitutive housekeeping genes, as well as inducible genes. Transcription factor Sp-1 (initially isolated from human HeLa cell lines) binds to some, but not all, hexanucleotide GGGCGG (GC box) sequences (10). Sp1-responsive promoters often contain multiple Sp-1 binding sites (11). A CACC box upstream of the human embryonic ε globin gene has also been shown to bind to Sp-1 *in vitro* and *in vivo* (12). Therefore, it has been repeatedly suggested that the T-C polymorphism of *CYP17* converting the sequence CACT into CACC may create such a site (8, 9) and provide a mechanism for higher level of expression of the variant *A2* allele. However, this possibility has never been verified experimentally.

Materials and Methods

Case and Control Population. Leukocyte DNA samples from a total number of 510 breast cancer patients were genotyped. Samples were collected from patients admitted to the Norwegian Radium Hospital, Ullevål Hospital (Oslo, Norway), and Haukeland Hospital (Bergen, Norway). The patient cohorts have been described previously (13, 14). Mean age at diagnosis for the total group was 59 (range, 27–91). Tumor and lymph node status were based on the pathology reports according to the WHO tumor-node-metastasis classification from 1988. The frequencies of the *CYP17* polymorphic alleles of the breast cancer patients were compared with the frequencies found in the series of 201 Norwegian control samples. The controls were healthy female individuals obtained through the Norwegian Population Registry as a population-based series of women ages 20–44.

Genotyping. Genotyping of the *CYP17*, 5′ UTR polymorphism was performed using forward primer 5′CATTCGCACCTCTGGAGTC3′ and reverse primer 5′GGCTCTTGGGGTACTTG3′ with PCR parameters, as described previously (8). PCR was performed in 25-μl reaction volumes on a Perkin Elmer 9600 thermocycler using 96-well microtiter plates. After the PCR reaction, restriction enzyme *MspAI* (2 units/reaction; Promega) and 10 × restriction enzyme buffer were added using a multichannel pipette directly to the microtiter plates to a final volume of 50 μl/well. Plates were incubated at 37°C overnight, and 10 μl of the restriction mix of all 96 samples were analyzed by standard electrophoresis on a single agarose gel, 3% NuSieve3:1 (FMC).

EMSA. The following DNA probes were used in this study:
*Sp-1-consensus: 5′GATCATATCTGCG***GGGCGG**GGCAGACACAG3′;
CYP17-A1^{20mer}: 5′CTTCTACT**C**<u>**CACT**</u>GCTGTCT3′;
CYP17-A2^{20mer}: 5′CTTCTACT**C**<u>**CACC**</u>GCTGTCT3′;
CYP17-A1^{100mer}:5′GTTGCCACAGCTCTTCTACT**C**<u>**CACT**</u>GCTGTCTATC-TTGCCTGCCGGCACCCAGCCACCATGTGGGAGCTCGTGGCTCTCTT-GCTGCTTACCCTAGCTTA3′;
CYP17-A2^{100mer}:5′GTTGCCACAGCTCTTCTACT**C**<u>**CACC**</u>GCTGTCT-ATCTTGCCTGCCGGCACCCAGCCACCATGTGGGAGCTCGTGGC-TCTCTTGCTGCTTACCCTAGCTTA3′;
ε-glob^{CCACC}: 5′ACCTGACT**C**<u>**CACC**</u>CCTGAGG3′ from Ref. 12;
ε-glob^{CCACT}: 5′ACCTGACT**C**<u>**CACT**</u>CCTGAGG3′ mutated.

Gel mobility shift assay was performed as described (15). Oligos for EMSA had been end-labeled with T4 polymucloetide kinase (Pharmacia Biotech, Uppsala, Sweden), annealed, and purified as described (15). One unit of recombinant Sp-1 protein (1 μl; Promega) was mixed with 1 pmol purified ^{32}P end-labeled DNA probe, 0.5 unit poly(dI-dC) (Pharmacia Biotech), and reaction buffer H, according to Oelgeschlager *et al*. (15), in a final volume of 20 μl. The binding reaction was allowed to proceed for 30 min either at room temperature

Received 3/1/99; accepted 4/29/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Norwegian Cancer Society and the Norwegian Research Council of Science and the Humanities.

[2] To whom requests for reprints should be addressed, at Institute for Cancer Research, the Norwegian Radium Hospital, Montebello 0310, Oslo, Norway. Phone: 47-22-93-44-17; Fax: 47-22-93-44-40; E-mail: nedelcheva.vessela@dnr.uio.no.

[3] The abbreviations used are: UTR, untranslated region; EMSA, electromobility shift assay.

or on ice. The reaction mixture was then run on a 5% polyacrylamide gel (1:37,5; BioRad) for approximately 1.5 h at 16°C. Protein-DNA complexes were visualized by autoradiography with intensifying screen overnight at −70°C.

Results

We have investigated the ability of motifs of *CYP17* 5′ UTR, containing the T-C polymorphic site, to bind to human transcription factor Sp-1 *in vitro*. ^{32}P end-labeled oligonucleotides, 20 bp long, corresponding to each of the alleles [*A1* (CACT) and *A2* (CACC)], were incubated with recombinant Sp-1 protein and analyzed by EMSA. The incubation mix was run on an EMSA gel and compared with the binding of the protein to an Sp-1 consensus sequence (GGGCCGG box). No binding of any of the motifs corresponding to the *CYP17* polymorphic alleles (*A1* or *A2*) was observed under the given experimental conditions (Fig. 1, *Lanes* 1–4) in contrast to a strong binding to the Sp-1 consensus sequence (GGGCCGG; Fig. 1, *Lanes* 7 and 8). To verify that under the same experimental conditions binding to sequence similar to that of the *A2* allele (CACC) can be observed, a sequence containing a CACC box, previously shown to bind transcription factor Sp-1 (12), was used as another Sp-1 consensus sequence. A 20-nucleotide long sequence from the human ε globin promoter, containing the CACC box (12), identical to the one found on the *A2* allele of *CYP17* (CACC), did bind Sp-1 under our experimental conditions (Fig. 1, *Lanes* 5 and 6 and Fig. 2, *Lanes* 1 and 2). It could be, therefore, concluded that the negative result in the case of *CYP17* was not due to failure in the experimental design. Furthermore, a single C-T substitution in the CACC box in the human ε globin promoter leading to the formation of a CACT motif, in analogy

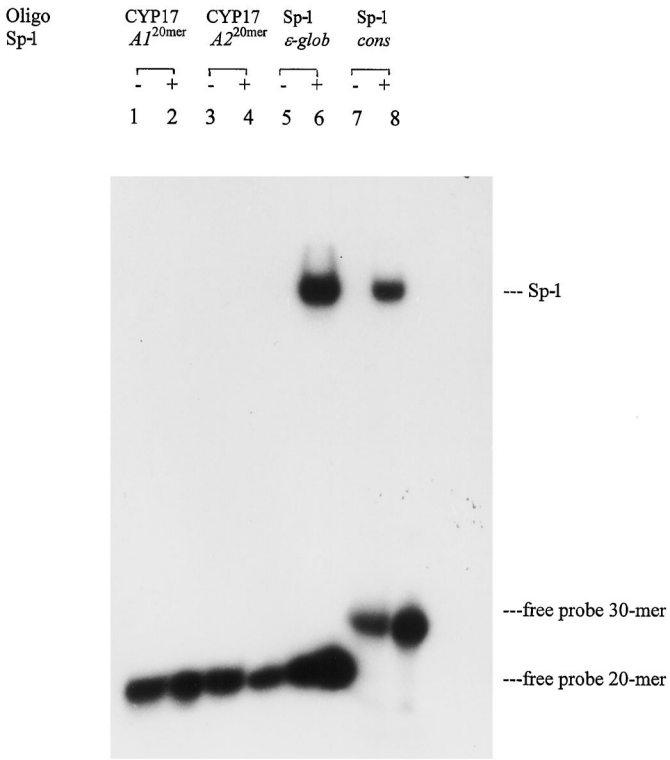

Fig. 1. EMSA of the polymorphic CYP17 5′ UTR. Purified ^{32}P end-labeled DNA probes were incubated with and without recombinant Sp-1 protein, (*Lanes 2, 4, 6* and *8* and *Lanes 1, 3, 5* and *7*, respectively). The binding reaction was allowed to proceed 30 min on ice. The reaction mixture was run on a 5% 1:37.5 polyacrylamide gel (BioRad) for approximately 1.5 h at 16°C. *Lanes* 1 and 2, *CYP17-A1*^{20mer}; *Lanes* 3 and 4, *CYP17-A2*^{20mer}; *Lanes* 5 and 6, Sp-1 *ε-glob*; *Lanes* 7 and 8, Sp-1cons.

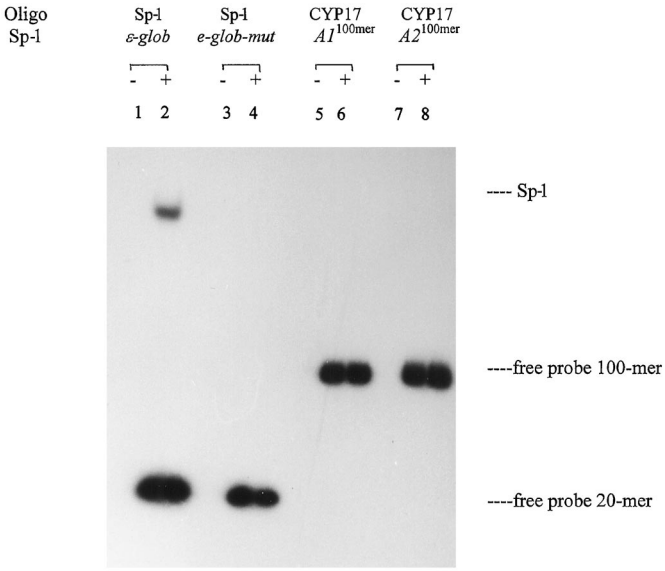

Fig. 2. EMSA of normal and mutated human ε globin promoter and a 100-bp long fragment of *CYP17* 5′ UTR, including the promoter sequence and polymorphic alleles. Purified ^{32}P-end-labeled DNA probes were incubated with and without recombinant Sp-1 protein, (*Lanes 2, 4, 6* and *8* and *Lanes 1, 3, 5* and *7*, respectively). *Lanes* 1 and 2, Sp-1 *ε-glob*; *Lanes* 3 and 4, Sp-1 *ε-glob*-mut; *Lanes* 5 and 6, *CYP17-A1*^{100mer}; *Lanes* 7 and 8, *CYP17-A2*^{100mer}.

CYP17
5' ***CTT CTA*** CT CCACC ***G***C T***GT CT*** 3'
5' ***ACC TGA*** CT CCACC ***C***C T***GA GG*** 3'
ε globin gene

Fig. 3. Alignment of the 20-nucleotide long sequence of the human ε-globin promoter with that of *CYP17*′ UTR.

to the *A1* allele of *CYP17*, abrogated this binding (Fig. 2, *Lanes* 3 and 4).

To examine the role of the sequences further upstream and downstream of the studied putative Sp-1 box in the *A2* allele, we repeated the experiment with a longer sequence from both alleles. Two different 100mers spanning from the promotor sequence 100 nucleotides downstream into the coding sequence, including several other putative CACC binding sites. None of these probes formed complexes with Sp-1 transcription factor (Fig. 2, *Lanes* 5–8).

Thus, given these experimental conditions, under which we were able to reproduce the Sp-1 binding to the ε globin promoter area from Yu *et al*. (12), we failed to observe an interaction between Sp-1 and the polymorphic *CYP17* 5′ UTR sequence. A comparison of the 20-nucleotide long sequence of the human ε-globin promoter with that of *CYP17* ′ UTR (Fig. 3) reveals a stretch of 12 nucleotides (ACTCCACCc/gCTG) of which 11, including the CACC box, are identical.

The genotype distribution of the *CYP17* polymorphism in Norwegian breast cancer patients and healthy controls is shown in Table 1. The allele frequencies in the control population, as well as in patients, did not deviate from Hardy-Weinberg equilibrium. No difference in the allele distribution was observed between cases and controls (P = 0.755). When analyzing the genotypes according to premenopausal (<45 years of age) and postmenopausal (>55 years of age), individuals homozygous for A2A2 were found to be almost twice as frequent in the older patients group (>55 years of age) compared with the younger patients (<45 years; 15.2% *versus* 8%, respectively).

Table 1 *Distribution of genotypes of the CYP17 5′UTR polymorphism in breast cancer patients and healthy controls. Relationship to stage of the disease and age of the patients at diagnosis.*

Genotype	Breast cancer patients (n = 510)	Controls (n = 201)	Age at diagnosis, years <45 (n = 75)	Age at diagnosis, years >55 (n = 257)	Stage of disease I+II (n = 272)	Stage of disease III+IV (n = 142)	Age + stage <45 and I+II (n = 54)	Age + stage >55 and III+IV (n = 93)
A1A1	202 (39.6%)	74 (36.8%)	27 (36.0%)	99 (38.5%)	108 (39.7%)	56 (39.4%)	19 (35.2%)	35 (37.6%)
A1A2	241 (47.3%)	101 (50.3%)	42 (56.0%)	119 (46.3%)	129 (47.4%)	66 (46.5%)	30 (55.6%)	41 (44.1%)
A2A2	67 (13.1%)	26 (12.9%)	6 (8.0%)	39 (15.2%)	35 (12.9%)	20 (20%)	5 (9.2%)	17 (18.3%)
	P = 0.76		P = 0.16		P = 0.94		P = 0.23	

This trend was in agreement with the data from Feigelson *et al*. (8), but the difference was not statistically significant (P = 0.16). The same nonsignificant trend was seen for patients >55 years at diagnosis with advanced stage of the disease. When comparing postmenopausal patients (>55 years of age) with stage III and IV to premenopausal patients with stage I and II (<45 years), A2A2 carriers were found more frequent among the older patients with advanced stage of disease (18.3% *versus* 9.2%). This difference was, however, not statistically significant (P = 0.23). There was no association between any of the *CYP17* genotypes and age of disease, lymph node status, and estrogen or progesterone status of the tumors (results not shown).

Discussion

No binding of human Sp-1 recombinant protein to a 20mer sequence containing the polymorphic site of *CYP17* was observed, although binding to another similar sequence in the ε-globin gene was demonstrated. The fact that a substitution of the last C in the CACC box in the ε-globin gene with a T abrogates the binding suggests that the CACC box is a necessary, although not sufficient determinant of Sp-1 binding. A comparison of the 20-nucleotide sequence of the human ε globin promoter with that of the *CYP17* 5′ UTR shows that the C immediately after the CACC box in ε-globin gene, which is a G at the same position in sequence of *CYP17*, is the most likely candidate to contribute to the observed difference. Because multimer links of Sp-1 had been described to act in synergism for which DNA looping is essential (11), the protein-DNA complex formation of a larger motif of 100 nucleotides, including both the polymorphic site and the promoter region, was studied. No complex formation of Sp-1 with these long stretches, containing further putative Sp-1 boxes, of either of the polymorphic sequences was observed.

The *A2* allele of *CYP17* has previously been found to be more frequent among postmenopausal women with advanced breast cancer compared with controls (8). In this study, a nonsignificant trend was observed for a higher frequency of the *A2A2* genotype among patients with age at diagnosis above 55 years *versus* patients with age at diagnosis below 45 years of age. When stratifying the patients similarly to previous studies (8), the same nonsignificant trend was observed for postmenopausal patients with higher stage of the disease (stage III and IV) *versus* premenopausal patients with stage I and II disease. The *A2A2* genotype is infrequent, which leads to small size of the compared groups and low statistical power. In addition, a very small portion of the patients in our cohort was with distant metastases (3.5%). If the *A2* allele is associated with a metastatic phenotype, as shown by Feigelson *et al*. (8), it would not have been detected in our study. Additional studies are necessary to clarify the association of the *A2* allele with metastatical breast cancer. Elevated estrogen levels throughout the premenopausal period was a suggested explanation of the observed association between the *A2* allele and a higher risk of advanced breast cancer in elderly patients (8). It was unclear why elevated estrogen levels should not confer a high-risk phenotype already earlier in life. While this manuscript was in preparation, other studies reported no association between the *CYP17* genotype and risk of breast cancer (16–18). However, these studies, discuss repeatedly the possible creation of Sp-1 site in the *A2* genotype, without testing this experimentally. We report a negative result. Additional studies are needed to find out whether the *A2* allele confers specifically a higher expression level of *CYP17*.

Cytochrome P450c17 is encoded by a single gene (*CYP17*) in mammals (6). The enzyme has a bifunctional active site: one catalytic center performs the 17α-hydroxylation of pregnenolone and progesterone and another, the 17,20-lyase activity, is responsible for the conversions of 17α-hydroxypregnenolone to dihydroepiandrosterone and 17α-hydroxyprogesterone to androstenedione, precursors of testosterone and estrogens (17, 18). In the adrenal glands the bulk of 17α-hydroxypregnenolone and 17OH progesterone is used in glucocorticoid production with minor fractions ending as difydroepiandrosterone and androstenedione (19). A single event of upregulation of transcription by Sp-1 binding, which was discussed as a possible phenotype of the T to C polymorphism of *CYP17*, would be expected to lead to up-regulation of both activities and have as a consequence overproduction of glucocorticoids. An interesting example is the failure of an aromatase inhibitor, aminoglutethimide, to block ovarian hormone production due to compensatory increase in the gonadotropin levels (20). The adrenal *CYP17* expresses considerable 17α-hydroxylase activity, but little 17,20 lyase activity, suggesting tissue-specific regulation of gene expression (2). Differential regulation of both activities is further supported by evidence that the 17,20 lyase activity, which yields precursors to testosterone and estradiol, is developmentally stimulated during puberty (19). Ovarian theca cells express high levels of 17, 20 lyase activity during the reproductive period, suggesting tissue specific regulation of *CYP17*. It would be of interest to search for interactions of the polymorphism in the 5′ flanking area of *CYP17* with tissue-specific transcription factors other than the relatively ubiquitous Sp-1, which are present in the ovarian theca cells, but absent in the adrenal tissues.

We report that the T-C polymorphism does not create a binding site for Sp-1. There was no observed difference in distribution of the different alleles among 201 healthy individuals and 510 breast cancer patients. Among the breast cancer patients, allelic distribution was not associated to age at onset of disease, tumor grade, lymph node status, stage, or expression of the estrogen and progesterone receptors.

References

1. Martucci, C. P., and Fishman, J. P450 enzymes of estrogen metabolism. Pharmacol. Ther., *57:* 237–257, 1993.
2. Voutilainen, R., and Miller, W. L. Developmental expression of genes for steroidogenic enzymes P450scc (20,22-desmolase), P450c17 (17 α-hydroxylase/17,20 lyase), and P450c21 (21-hydroxylase) in the human fetus. J. Clin. Endocrinol. Metab., *63:* 1145–1150, 1986.
3. Thomas, H. V., Key, T. J., Allen, D. S., Moore, J. W., Dowsett, M., Fentiman, I. S., and Wang, D. Y. A prospective study of endogenous serum hormone concentrations and breast cancer risk in premenopausal women on the island of Guernsey. Br. J. Cancer, *75:* 1075–1079, 1997.
4. Hankinson, S. E., Willett, W. C., Manson, J. E., Colditz, G. A., Hunter, D. J., Spiegelman, D., Barbieri, R. L., and Speizer, F. E. Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. J. Natl. Cancer Inst., *90:* 1292–1299, 1998.

5. Hiegelson, H. S., and Henderson, B. E. Estrogens and breast cancer. Carcinogenesis (Lond.), *17:* 2279–2284, 1996.
6. Picardo-Leonard, J., and Miller, W. L. Cloning and sequence of the human gene for p450c17 (steroid 17a-hydroxylase/17,20 lyase): similarity with the gene for P450c21. DNA, *6:* 439–448, 1987.
7. Carey, A. H., Chan, K. L., Short, F., White, D., Williamson, R., and Franks, S. Evidence for a single gene effect causing polycistic ovaries and male pattern baldness. Clin. Endocrinol., *38:* 653–658, 1993.
8. Feigelson, H. S., Coetzee, G. A., Kolonel, L. N., Ross, R. K., and Henderson, B. E. A polymorphism in the *CYP17* gene increases the risk of breast cancer. Cancer Res., *57:* 1063–1065, 1997.
9. Feigelson, H. S., Shames, L. S., Pike, M. C., Coetzee, G. A., Stanczyk, F. Z., and Henderson, B. E. A cytochrome p450c17a gene (*CYP17*) polymorphism is associated with serum estrogen and progesterone concentrations. Cancer Res., *58:* 585–587, 1998.
10. Kadonada, J. T., Carner, K. R., Masiarz, F. R., and Tjan, R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell, *51:* 1079–1090, 1987.
11. Gidoni, D., Dynan, W. S., and Tjan, R. Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature (Lond.), *312:* 409–413, 1984.
12. Yu, C. Y., Motamed, K., Chen, J., Bailey, A. D., and Shen, S. K. J. The CACC box upstream of human embryonic ε-globin gene binds Sp1 and is a functional promoter element *in vitro* and *in vivo*. J. Biol. Chem., *14:* 8907–8915, 1991.
13. Nedelcheva Kristensen, V., Andersen, T. I., Lindblom, A., Nesland, J., Olsen, A., and Børresen-Dale, A-L. A rare CYP19 (aromatase) variant increases the risk of breast cancer. Pharmacogenetics, *8:* 43–48, 1998.
14. Bukholm, I. K., Nesland, J., Kåresen, R., Jacobsen, U., and Børresen-Dale, A-L. Relationship between abnormal p53 protein and failure to express p21 protein in human breast carcinomas. J. Pathol., *181:* 140–145, 1997.
15. Oelgeschlager, M., Nuchprayoon, I., Luscher, B., and Friedman, A. D. C/EBP, c-Myb and PU.1 cooperate to regulate the neutrophil elastase promoter. Mol. Cell. Biol., *16:* 4717–4725, 1996.
16. Dunning, A. M., Healey, C. S., Pharoah, P. D. P., Foster, N. A., Lipscombe, J. M., Redman, K. L., Easton, D. F., Day, N. E., and Ponder, B. A. J. No association between a polymorphism in the steroid metabolism gene *CYP17* and risk of breast cancer. Br. J. Cancer, *77:* 2045–2047, 1998.
17. Weston, A., Pan, C-f., Bleiweiss, I. J., Ksieski, H. B., Roy, N., Maloney, N., and Wolf, M. S. *CYP17* genotype and breast cancer risk. Cancer Epidemiol. Biomark. Prev., *7:* 941–945, 1998.
18. Helzlsouer, K. J., Huang, H-Y., Strickland, P. T., Hoffman, S. H., Alberg, A. J., Comstock, G. W., and Bell, D. Association between CYP17 polymorphisms and the development of breast cancer. Cancer Epidemiol. Biomark. Prev., *7:* 945–951, 1998.
19. Nakajin, S., Shinoda, M., Haniu, M., Shively, J. E., and Hall, P. F. C21 steroid side chain vleavage enzyme from porcine adrenal microsomes: purification and characterization of the 17a hydroxylase/C17,20-lyase cytochrome P450. J. Biol. Chem., *259:* 3971–3976, 1984.
20. Santen, R. J., Samojlik, E., and Wells, S. A. Resistance of the ovary to blockade of aromatization with aminogluthimide. J. Clin. Endocrinol. Metab., *51:* 473–477, 1980.

CYP17 and Breast Cancer Risk: The Polymorphism in the 5′ Flanking Area of the Gene Does Not Influence Binding to Sp-1

Vessela Nedelcheva Kristensen, Ellen K. Haraldsen, Kristin B. Anderson, et al.

Cancer Res 1999;59:2825-2828.