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Doris Auer, Daniel Reimer, Verena Porto, Martina Fleischer, Julia Roessler, et al.. Expression of coxsackie-adenovirus receptor is related to estrogen sensitivity in breast cancer. *Breast Cancer Research and Treatment*, 2008, 116 (1), pp.103-111. 10.1007/s10549-008-0108-0 . hal-00478247

HAL Id: hal-00478247

<https://hal.science/hal-00478247>

Submitted on 30 Apr 2010

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Expression of coxsackie-adenovirus receptor is related to estrogen sensitivity in breast cancer

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Received: 14 March 2008 / Accepted: 18 June 2008 / Published online: 10 July 2008
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Abstract This study analyzes the relationship between coxsackie-adenovirus receptor (CAR) expression (transmembrane and soluble isoforms) and hormone sensitivity in 95 breast cancers. Furthermore, prognostic significance of the expression of the various CAR isoforms was investigated. In addition, inducibility of CAR expression by estradiol and tamoxifen was assessed in various breast cancer cell lines. Expression of transmembrane CAR (hCAR) highly correlated with estrogen receptivity, but was independent of the expression of progesterone receptor (PR). Furthermore, hCAR expression was significantly higher in tumors with low-grade malignancy. However, no relationship between hCAR expression and tumor size, lymph node status, or survival was revealed. In the hormone receptor-positive breast cancer cell line T47-D expression of hCAR and its soluble isoforms was increased by treatment with estradiol and tamoxifen. In contrast, no induction of either CAR isoform was achieved in receptor-negative cell lines. Furthermore, enhancement of hCAR expression was significantly greater when cells were treated with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) than when treated with estradiol or tamoxifen. Moreover, sensitivity to TSA induction of hCAR was considerably greater in receptor-positive than in receptor-negative cell lines. No additive effect on CAR expression was found when TSA was combined with either estradiol or tamoxifen. In conclusion, the so far undescribed association between estrogen receptivity and the expression of hCAR in breast cancer seems to

not only reflect a phenotype of low malignancy, but expression of hCAR may also be directly influenced by ER-specific ligands.

Keywords Breast cancer · Coxsackie-adenovirus receptor · Estrogen receptor · HDAC inhibitor · Hormone sensitivity · Tamoxifen

Introduction

The coxsackie-adenovirus receptor (CAR) is considered essential for cell attachment and cell entry of the coxsackie B and adenovirus. During the past decade CAR was extensively studied in the context of adenovirus-based gene therapy. Moreover, in a number of tumor entities CAR expression has been found to correlate inversely with the biologic aggressiveness of tumors. In bladder cancer, for example, the loss of CAR expression is associated with established markers of biologically aggressive bladder transitional cell carcinoma such as lymph node metastases, stage and high pathologic grade [1].

CAR has also been shown to be physically associated with epithelial tight junctions and involved in cell–cell adhesion. In polarized epithelial cells, CAR is co-localized with the tight junctions, where it associates with ZO-1 and contributes to the barrier functions in the circulation of macromolecules and ions [2].

CAR is a 46-kDa integral membrane protein with a typical transmembrane domain, a long cytoplasmic domain, and an extracellular domain composed of two immunoglobulin-like domains [2] and belongs to the immunoglobulin superfamily [3]. The human CAR gene is located on chromosome 21q11.2 and consists of at least eight exons distributed over an area of 54 kb. Beside the

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transmembrane CAR isoform (hCAR), the skipping of exons III–VI in CAR 2/7, exons IV–VI in CAR 3/7, and exons V–VI in CAR 4/7 results in at least three soluble CAR splice variants (CAR 2/7, CAR 3/7, CAR 4/7) that contain the leading sequence encoded by exon I, different portions of the immunoglobulin domains, but lack the transmembrane domain [4].

The role of membranous CAR in tumor progression and metastasis seems to differ among various tumor entities and remains the subject of debate. Several studies performed in human tumors, including prostate cancer, bladder cancer, and glioblastoma multiform, indicate a significant reduction in CAR expression during tumor progression [5–8]. In bladder cancer cell lines CAR was shown to be involved in modulating cell cycle regulators, such as p21^{CIP} and Rb, suggesting a possible role of CAR as a tumor suppressor [6]. Furthermore, it has been shown that the retroviral transfection of CAR into the primarily CAR-deficient human bladder cancer cell line T24 causes to growth inhibition [9]. The same effect was observed in CAR-deficient human prostate cancer and malignant glioma cells [5, 8]. However, in breast cancer, Martin et al. [10] reported that CAR expression was found to be higher in metastatic tumors than in early breast cancers. Furthermore, these authors also found that tumors with nodal involvement were associated with higher levels of CAR. On the other hand, tumors from patients with local recurrences exhibited reduced CAR expression, even in comparison with those cases that remained disease-free over the follow-up period. Moreover, CAR expression increased with higher grade of malignancy, but did not reach statistical significance. In survival analysis, high CAR expression was revealed to be significantly related to poor overall survival in breast cancer patients [10].

In ovarian cancer, however, no prognostic relevance was pointed out for the membranous form of CAR, but expression of the soluble CAR isoforms CAR 3/7 and 4/7 was shown to be an independent prognosticator predicting unfavorable progression-free (CAR 4/7) and overall survival (CAR 3/7 and CAR 4/7) [11].

Interestingly, in human ovarian cancer cell lines (PEO4, PEO1), CAR expression was enhanced and transduction efficacy by adenoviral vectors was increased by 17 β -estradiol treatment [12]. For that reason and because of the major role of steroid hormones in the biology of breast cancer, we examined whether a relationship exists between CAR expression and hormone sensitivity in cancers of the breast. In addition, the expression of membranous CAR (hCAR) and its soluble isoforms (CAR 2/7, CAR 3/7, and CAR 4/7) was investigated for prognostic significance in a set of 95 breast cancer patients. In order to investigate a possible functional relationship between steroid hormones and the family of CAR isoforms, *in vitro* assays of CAR

inducibility by 17 β -estradiol and 4-hydroxy-tamoxifen were performed in hormone receptor-positive and -negative breast cancer cell lines.

Materials and methods

Patients

Tissue samples from patients with breast cancer were collected during primary surgery at the Department of Obstetrics and Gynecology in Innsbruck ($n = 95$). Breast tissue samples obtained from patients who underwent esthetic surgery for other than inflammatory or malignant conditions served as control ($n = 11$). All included patients gave written consent for tissue use in research, and the study was approved by the local Institutional Ethics Review Board. Tissues were immediately frozen and stored at -80°C .

All patients ($n = 95$) were monitored within the out-patient follow-up program of the Department of Obstetrics and Gynecology, Innsbruck Medical University, and the median observation period of the included patients was 92 (52–130) months. Clinicopathologic characteristics are summarized in Table 1.

Estrogen and progesterone receptor levels were obtained from patient charts and were routinely determined by immunohistochemistry using the commercially available ERICA and PRICA kits (Abbott, Wiesbaden, Germany). Receptor status was recorded as percentage of positive staining cells (0–100%) and as staining intensity (1–3) for both ER and PR. Samples with a percentage ≥ 10 were considered receptor-positive. HER2/neu status was analyzed immunohistochemically using the HercepTest (DAKO, Denmark A/S, Glostrup, Denmark). Membrane immunoreactivity and membrane staining patterns were evaluated and scored using the 0 to 3+ scoring system according to the manufacturer's protocol. Tumors with a HER-2/neu score of 2+ in immunohistochemical analysis were also tested for HER-2/neu gene amplification using fluorescence *in situ* hybridization (FISH) and the PathVysion kit (Vysis, Downers Grove, IL). According to the HER-2/neu testing recommendations of the American Society of Clinical Oncology and the College of American Pathologists, tumors were classified as positive when the immunohistochemical score was 3+ or gene amplification was detected by means of *in situ* hybridization [13].

Cell lines

Established breast cancer cell lines MCF-7, T-47D, MDA-MB-361, Zr75-1, 734-B, BT-20 and MDA-MB-231 obtained from ATCC (American Type Culture Collection, Maryland,

Table 1 Clinicopathologic characteristics

	<i>n</i>	Median	Q_1-Q_3
Age (years)	95	60.3	48.6–72.0
Median DFS (m)		84.0	37.0–131.0
Median OAS (m)		92.0	53.8–130.3
	<i>n</i>	%	
Death during follow-up	49/95	51.6	
Relapse during follow-up	93		
Relapse	43/93	46.2	
Local relapse	14/93	15.1	
Systemic relapse	36/93	38.7	
pT	94		
pT-1	20	21.3	
pT-2	56	59.6	
pT-3	10	10.6	
pT-4	8	8.5	
pN	88		
pN-0	31	35.2	
pN-1	56	63.6	
pN-2	1	1.2	
Histology	95		
Ductal	78	82.1	
Lobular	12	12.6	
Medullary/mucinous	5	5.3	
Grade of malignancy	93		
1	27	29.0	
2	54	58.1	
3	12	12.9	
Estrogen receptor	95		
Negative	31	32.6	
Positive	64	67.4	
Progesterone receptor	93		
Negative	34	36.6	
Positive	59	63.4	
HER2/neu	88		
Negative	60	68.2	
Positive	28	31.8	
Menopausal state	95		
Pre	30	31.6	
Post	65	68.4	
M1	93		
M0	85	91.4	
M1	8	8.6	

USA) were cultured in appropriate medium for 24 h. The original medium was displaced by MEM without phenol red (Gibco) supplemented with 10% charcoal/dextran-treated FBS (HyClone). Each of the cell lines examined was treated with 10^{-10} M 17β -estradiol (estradiol) alone, 10^{-6} M 4-hydroxy-tamoxifen (tamoxifen) alone,

10^{-10} M 17β -estradiol plus 10^{-6} M 4-hydroxy-tamoxifen, transforming growth factor (TGF) -alpha (10 ng/ml), TGF-beta (10 ng/ml), and epidermal growth factor (EGF) (10 ng/ml) for 72 h. Furthermore, cells were treated with trichostatin A (TSA) (100 ng/ml) for 24 h alone, 2.5 μ M 5-aza-2' deoxycytidine (5-AZA) for 48 h alone, 10^{-10} M 17β -estradiol alone or in combination with these agents.

RNA extraction and RT reaction

Total RNA was isolated from tissue samples or cultured cells using the guanidium thiocyanate-phenol-chloroform method according to the manufacturer's protocol (RNA-gents® Total RNA Isolation System, Promega, Madison, WI). Integrity was evaluated by assessing the 18S- and 28S-ribosomal RNA bands in 1% ethidium bromide-stained agarose gels. DNase treatment and reverse transcription were performed as described recently [11].

Primers and probes

Specific primers and probes for hCAR, its soluble splice variants CAR 2/7, CAR 3/7, CAR 4/7 and for TBP (TATA box-binding protein; a component of the DNA-binding protein complex TFIID as an endogenous RNA control) were determined with the computer program "Primer Express" (Applied Biosystems, Foster City, CA). To prevent amplification of contaminating genomic DNA, the forward primers were placed at a junction between two exons. The various splice variants were distinguished by placing the forward primer between exon 2 and exon 7 for CAR 2/7, exon 3 and exon 7 for CAR 3/7, exon 4 and exon 7 for CAR 4/7 and between exon 6 and exon 7 for hCAR. The reverse primer (CAR Ex7 AS) and the probe (CAR Ex7 Taq) used for all splice variants were placed in exon 7. Sequences of primers and probes are given in Table 2.

Real-time PCR amplification

RT-PCRs were performed using an ABI Prism 7900 Detection System (Applied Biosystems, Foster City, CA) in a total volume of 25 μ l reaction mixture containing 5 μ l of each appropriately diluted RT sample (standard curve points and test samples), 12.5 μ l TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nm of each primer and 250 nm of the probe. Cycling conditions were an initial step at 50°C for 2 min, a denaturing step at 95°C for 10 min, and 45 cycles at 95°C for 15 s and 65°C for 1 min. Real-time PCR efficiencies were determined by amplifying serially diluted cDNA isolated from the breast cancer cell line SKBR-3. Real-time PCR assays were conducted in duplicate and the mean value was used for calculation. Only PCR experiments with a

Table 2 Primers and probes of real-time PCR

Isoform	Exon	Oligonucleotide	Sequence
CAR 2/7_S	2/7	Forward primer	5'-GGT GGA TCA AGT <u>GGG</u> AAG ATG T-3'
CAR 3/7_S	3/7	Forward primer	5'-TCT GGT AGT TCT <u>TGG</u> GAA GAT GTG-3'
CAR 4/7_S	4/7	Forward primer	5'-CAT GGT TAG CAG <u>GGA</u> AGA TGT G-3'
CAR_AS	7	Reverse primer	5'-TGA ATG ATT ACT GCC GAT GTA GC-3'
CAR_Taq	7	Probe	5'-FAM-CAC CTC CAA AGA GCC GTA CGT CCA CTG-TAMRA-3'
hCAR_S	6	Forward primer	5'-AGT GAA GAC TTT GAA CGC ACT CC-3'
hCAR_AS	7	Reverse primer	5'-CGC ACC CAT TCG ACT TAG ATT AG-3'
hCAR_Taq	6/7	Probe	5'-FAM-CAG AGT CCG ACT CTC CCA CCT GCT AAG -TAMRA-3'

Exon/exon boundaries are underlined. The reverse primer and probe are located in exon 7, whereas various splice variants are defined by specific forward primers

detection efficiency more than 95% were used for statistical analysis ($R_0 > 0.99$). Gene expression levels were determined with the Comparative C_T method according to User Bulletin 2 (Applied Biosystems, Foster City, CA). Levels of CAR transcripts detected in patient samples were normalized to TBP.

Immunohistochemical demonstration of membranous hCAR

Immunohistochemistry was performed on paraffin-embedded 4 μ m sections using the UltraView Universal DAB Detection Kit (Ventana, Tucson, AZ, USA) based on a mouse anti-rabbit Ventana BenchMark automated slide staining system. Antigen-retrieval was performed with cell conditioning solution 1 (standard); slides were incubated for 1 h at 37°C with the rabbit polyclonal anti-CAR H-300 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used as primary antibody (dilution 1:50). Counterstaining was attained using Nexes Hematoxylin, followed by Nexes Bluing Reagent. Negative controls were obtained by omitting the primary antibody.

Western blot analyzes

Extracts were prepared by lysing cell pellets (5×10^{-6} cells) in Co-IP buffer (Co-IP 50 mM HEPES NaOH pH 7.5; 1% Triton X-100; 150 mM NaCl; 0.5 mM EDTA; 10% glycerol) with 1% protease inhibitor (HALT Protease Inhibitor Cocktail, Pierce Biotechnology, Rockford, IL, USA). Cell pellets were resuspended in Co-IP buffer and incubated on a shaker at 4°C for 1 h. Extracts were clarified by centrifugating at 17,000g for 15 min at 4°C.

For Western blot analysis, total protein (60 μ g) was separated on ready Tris-HCl gel, 4–20% (Bio Rad) and blotted onto nitrocellulose membranes. Membranes were incubated with rabbit polyclonal anti-CAR (1:200; H-300,

Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-GAPDH (1:1,000; Chemicon) antibodies followed by HRP-conjugated secondary antibodies.

Blots were exposed to ECL plus Western blotting detection reagents (Amersham/GE Healthcare) and detected by photo imager.

Statistics

Differences between expression levels of CAR transcripts in cell culture experiments were assessed with the Student's *t*-test. For analysis between patient subgroups regarding, for example, hormone receptor, lymph node status or grade of malignancy we used either the Kruskal-Wallis or the Mann-Whitney *U* non-parametric test. Survival analyses for disease-free survival and overall survival were conducted with the Kaplan-Meier method and differences between groups were determined with the log-rank test.

Results

mRNA expression of CAR isoforms related to clinicopathologic parameters

Expression levels of hCAR mRNA and that of its soluble splice variants CAR 2/7, CAR 3/7, and CAR 4/7 were assessed in breast cancer samples of 95 patients and 11 normal breast tissue samples. Soluble CAR 2/7 and CAR 3/7 isoforms, were not detectable in four and three cancers, respectively.

mRNA of CAR 2/7, CAR 3/7, and CAR 4/7 isoforms was expressed to a higher extent in controls than in tumor samples (Table 3). Between controls and tumors no significant difference in hCAR expression was observed. Expression of the soluble CAR isoforms CAR 2/7, 3/7, and

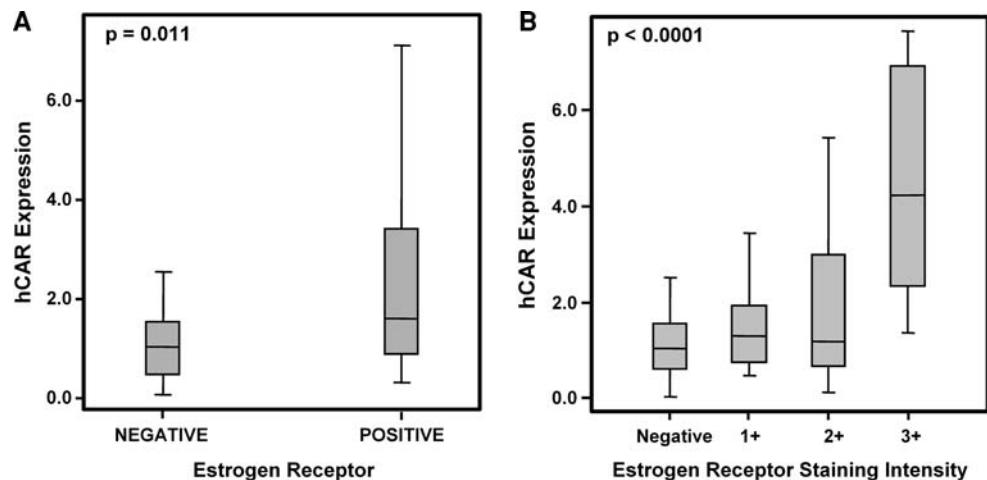
Table 3 CAR expression in breast cancer samples and healthy controls

Breast cancer samples	Controls				P-value	
	Median	(Q_1 – Q_3)	Median	(Q_1 – Q_3)		
hCAR	1.239	0.363–2.114	hCAR	1.856	1.456–2.256	0.17
CAR2/7	0.496	0.046–0.945	CAR2/7	1.171	0.722–1.619	0.03
CAR3/7	0.405	0.004–0.807	CAR3/7	0.937	0.527–1.347	0.07
CAR4/7	0.672	0.289–1.054	CAR4/7	1.161	0.836–1.486	0.03

4/7 correlated with tumor histology as these isoforms were expressed to a significantly lower level in ductal and lobular carcinomas than in medullary/mucinous carcinomas ($P < 0.015$, $P < 0.005$ and $P < 0.015$, respectively). Moreover, the membranous isoform was expressed to a higher extent in tumors with low-grade malignancy [median value 2.18 (Q_1 1.13 and Q_3 3.63)] than in tumors with a higher grade of malignancy [median value 1.05 (Q_1 0.68 and Q_3 1.91)] ($P < 0.004$). HER2/neu-positive tumors showed a tendency toward decreased hCAR transcript levels [median value 1.00 (Q_1 0.64 and Q_3 2.36)] in comparison to HER2/neu-negative tumors [median value 1.45 (Q_1 1.00 and Q_3 2.94)]. In the subgroup of tumors qualified as steroid receptor-positive ($n = 55$) this difference was more prominent, in terms that HER2/neu negativity was associated with significantly higher hCAR expression [median value 1.72 (Q_1 0.20 and Q_3 7.68)] as compared with HER2/neu positivity [median value 0.87 (Q_1 0.26 and Q_3 3.90)]; ($P < 0.03$).

Between patients with and without lymph node involvement no significant difference in CAR expression (membrane and soluble isoforms) was observed, and no reliable association between tumor size and CAR expression was observed. Furthermore, survival analyses did not reveal a prognostic significance for expression of the various CAR isoforms in terms of either disease-free or overall survival.

Fig. 1 Expression of membranous hCAR in relationship to the ER status: (a) Breast cancers dichotomized in ER-positive and -negative tumors. (b) Subdivision on ER staining intensity



mRNA levels of CAR isoforms related to the hormone receptor status

Estrogen receptor (ER)-positive tumors showed increased hCAR transcript levels [median value 1.67 (Q_1 0.80 and Q_3 3.49)] in comparison to ER-negative tumors [median value 1.05 (Q_1 0.61 and Q_3 1.69)] ($P = 0.011$) (Fig. 1a). A closer look at the immunohistochemical results obtained for steroid receptors revealed that especially ER-positive tumors with a high staining intensity were found to be strongly associated with increased hCAR expression ($P < 0.0001$) (Fig. 1b). PR-positive tumors showed a tendency to higher hCAR expression [median value 1.31 (Q_1 0.85 and Q_3 2.49)] than did PR-negative tumors [median value 1.02 (Q_1 0.53 and Q_3 2.16)], but these differences did not reach statistical significance ($P = 0.065$). When the subgroup of ER-positive tumors was compared to ER-negative/PR-positive and ER-negative/PR-negative tumor subgroups, we observed that elevated hCAR expression was strictly associated with ER expression and was independent of tumor PR expression (Table 4). However, no consistent association between the expression of the mRNA of any soluble CAR isoform and steroid hormone receptivity was observed. As depicted in Fig. 2a and b, the close relationship between the membranous hCAR and ER was corroborated when both molecules were demonstrated by immunohistochemistry.

Inducibility of CAR isoforms by estradiol and tamoxifen

In order to explore whether the revealed correlation between CAR and ER expression in the breast cancer samples analysed, simply reflects the grade of tumor differentiation or whether this correlation is based on a functional link between both molecules, we evaluated the inducibility of hCAR and its soluble isoforms by estradiol or tamoxifen in vitro. When

Table 4 hCAR expression in ER-/PR-positive tumors

	ER-positive	ER-negative PR-positive	ER-negative PR-negative	
Median	1.672	1.185	0.999	$P < 0.04$
Q_1 – Q_3	0.796–3.486	0.751–1.448	0.582–1.847	

hormone receptor-positive and -negative cell lines were compared, a significantly higher constitutive expression of hCAR and its soluble isoforms was observed in the hormone-sensitive cell lines ($P < 0.03$).

In the hormone receptor-positive cell line T47-D, treatment with estradiol significantly induced the expression of membranous and soluble CAR isoforms ($P < 0.05$). Significant inducibility of all CAR isoforms was observed to a lesser extent when cells were treated with tamoxifen or the combination of tamoxifen and estradiol (Fig. 3). These effects were also found in the other hormone receptor-positive cell lines analysed, but in those cell lines the differences did not reach statistical significance. The revealed effects of steroid and tamoxifen treatment were not found to be confined to the mRNA level but were also evidenced at the protein level by means of Western Blot analyzes (Fig. 4). Of note is the fact that in the hormone receptor-positive cell line MCF-7 no constitutive expression of any

CAR isoform was detectable and CAR expression was not inducible with estradiol or tamoxifen treatment.

Mechanisms possibly involved in the estrogen and tamoxifen induction of CAR isoforms

As no responsive element for ER was found in the promoter of CAR using the transcription factor-binding profile database JASPAR (<http://jaspar.cgb.ki.se>), we wanted to explore whether CAR expression is induced in an auto- or paracrine manner by factors known to be up-regulated by estrogens or tamoxifen in hormone-sensitive breast cancer cells. However, treatment of T47D cells with TGF-beta, TGF-alpha or EGF virtually did not affect the expression of hCAR and tended to decrease the transcripts of the soluble isoforms (data not shown).

To explore whether epigenetic phenomena are potentially involved in the ER-dependent induction of the expression of the various CAR isoforms, cell lines were furthermore treated with the histone deacetylase (HDAC) inhibitor TSA and the DNA methyltransferase inhibitor 5-AZA. In six of the seven cell lines tested, the combination of TSA and 5-AZA led to a highly significant 2.3- to 3.5-fold up-regulation of hCAR-specific transcripts ($P < 0.01$) (Fig. 5). This effect was less prominent when cells were

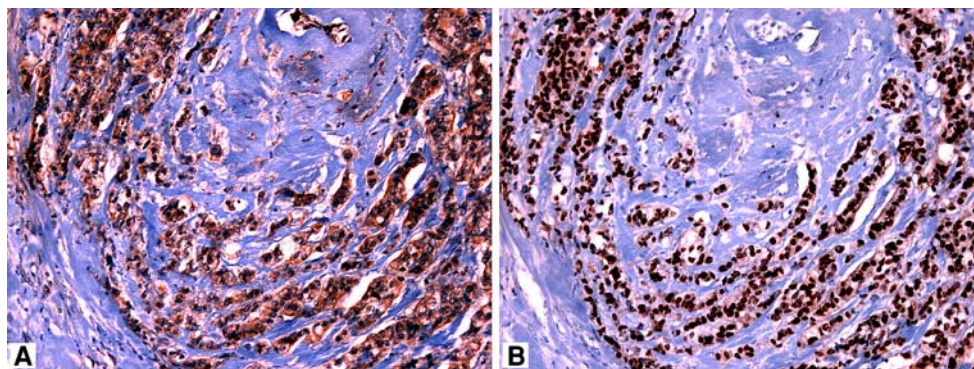
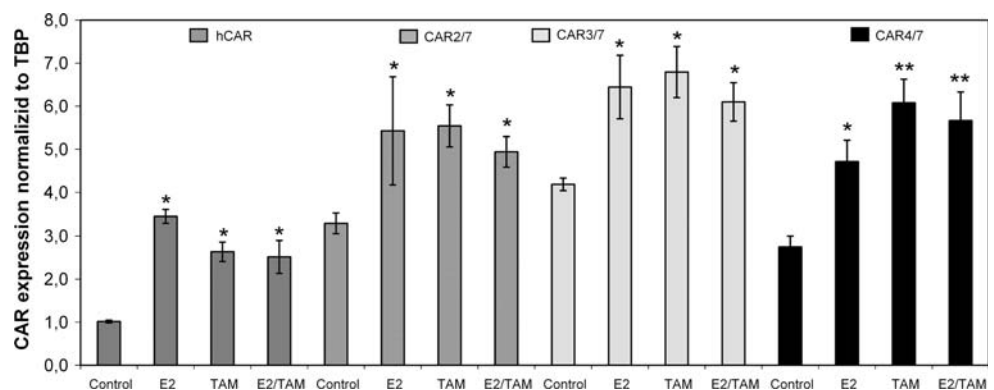


Fig. 2 Immunohistochemical concordant expression of membranous hCAR and ER in a breast cancer sample: (a) high membranous immunostaining of hCAR. (b) High nuclear ER staining intensity

Fig. 3 Expression of hCAR and its soluble isoforms in T47-D cells treated with E2, tamoxifen or a combination of both. * $P < 0.05$; ** $P < 0.01$ in comparison with the respective control



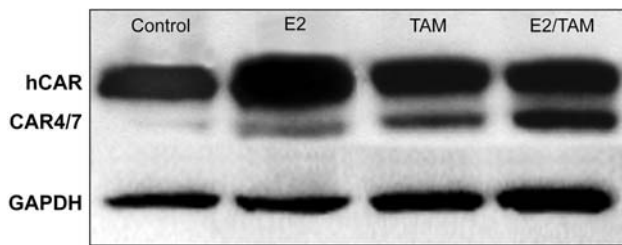


Fig. 4 Expression of hCAR and soluble CAR 4/7 isoform at the protein level after exposure of T47-D cells to E2, tamoxifen, or both

treated with TSA alone (1.8- to 2.5-fold) ($P < 0.05$) and was completely missing when cells were treated with 5-AZA alone. Interestingly, sensitivity to TSA was significantly higher in receptor-positive as compared to receptor-negative cell lines ($P < 0.03$). Inducibility of the soluble isoforms paralleled that observed for membranous CAR and was of the same magnitude. It is worth to note that neither TSA, 5-AZA, nor a combination of both agents, was able to induce demonstrable CAR expression in MCF-7 cells that consequently should be regarded as a CAR-deficient breast cancer cell line.

Discussion

Steroid hormone receptors play a critical role in the development and progression of breast cancer. Most importantly, the expression of ER and/or PR by tumor cells provides important information that is critical for the choice of treatment [14]. In addition, the expression of steroid hormone receptors correlates with well-differentiated tumors [15].

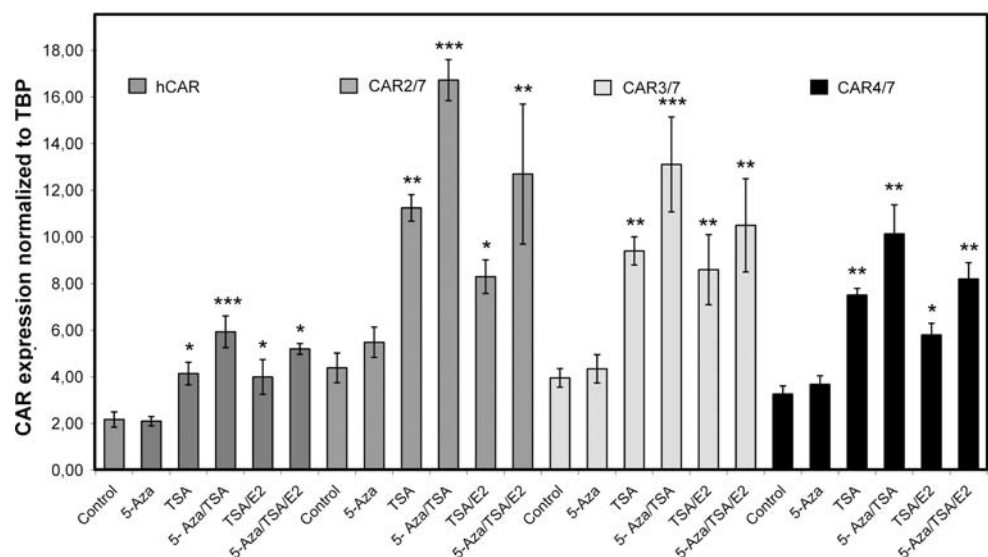
CAR, which was intensively studied during the last decade in the context of adenoviral-based gene therapy, has

also been identified as a molecule associated with low malignant phenotype in urogenital cancers [1]. Even more, membranous CAR was implicated in cell cycle control and therefore considered to be a molecule with tumor-suppressing properties [6].

Indeed, in our series of breast cancers, hCAR correlated with the grade of malignancy. Moreover, HER2/neu-positive tumors tended to show decreased hCAR expression as compared to HER2/neu-negative tumors. Although these findings were not true for the soluble CAR isoforms, it seems that high hCAR expression is associated with well-differentiated tumors. However, no association with lymph node status or tumor size was observed, and neither for membranous nor for soluble CAR isoforms was a prognostic significance in terms of disease-free or overall survival revealed. In this regard our findings disagree with those reported by Martin et al. [10], who found poor overall survival for breast cancer patients with elevated transmembrane CAR levels.

Findings showing that expression of membranous CAR in ovarian cancer cell lines (PEO4, PEO1) can be induced with estradiol [12] prompted us to investigate whether an association exists between the expression of this membranous receptor or its soluble isoforms and hormone sensitivity in breast cancer. In the breast cancers analysed, we observed that high expression of transmembrane CAR is strongly related to estrogen but not to progesterone receptivity of tumors. Furthermore, hCAR expression was higher in receptor-positive breast cancer cell lines than in receptor-negative cell lines. These findings would primarily suggest that hCAR is associated with tumors exhibiting a highly differentiated phenotype, which are otherwise known to be highly sensitive for steroid hormones. However, the performed in vitro analyzes show that membranous as well as soluble CAR isoforms can be

Fig. 5 Expression of hCAR and its soluble isoforms in T47-D cells after treatment with DNA-methyltransferase (DNMT) inhibitor 5-AZA, HDAC inhibitor TSA either alone or in combination. TSA and 5-AZA treatment was performed with and without E2. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to the respective control



induced in receptor-positive T-47D cells with either estradiol or tamoxifen. This was also the case, even to a lesser extent and with borderline statistical significance, for other receptor-positive cell lines, but was not observed in receptor-negative cell lines. Thus, theoretically it is possible that CAR expression is directly influenced by estradiol or by selective estrogen receptor modulators such as tamoxifen. However, screening of the putative CAR promoter region revealed no binding site for ER, but identified specific binding sites for SP-1 and the E2F transcription factor family. Earlier observations that expression of E2F transcription factors is increased following EGF stimulation [16] prompted us to elucidate whether CAR up-regulation by estradiol could be driven in an autocrine or paracrine manner involving TGF- α , which is a ligand of the EGF receptor and has been shown to be released in enhanced amounts following estradiol stimulation [17]. Moreover, in ER-positive breast cancer cells tamoxifen has been found to increase the release of TGF- β [18] which could be a further candidate with the potential to influence CAR expression. Yet, in our in vitro model hCAR expression remained unaffected and the transcripts of the soluble isoforms even tended to be decreased by EGF, TGF- α , and TGF- β treatment.

As CAR expression is known to be under tight epigenetic control, particularly by histone acetylation, and the phytoestrogen genestein was shown to inhibit HDAC activity in T24 bladder cancer cells [19], we were tempted to speculate that the stimulating effect of estradiol on the CAR gene could be mediated by epigenetic changes. We found a significant induction of hCAR transcripts when T-47D cells were treated with TSA alone or in combination with 5-AZA, but no effect was seen when cells were treated with 5-AZA alone. This corroborates the notion that the CAR gene is more likely regulated by histone acetylation than by methylation of its promoter CpG islands. Furthermore, the stimulatory effects of TSA on the expression of CAR were by far more pronounced than those observed for estradiol or tamoxifen treatment. In addition, a much lower sensitivity to TSA regarding CAR induction was revealed for the ER-negative versus ER-positive cell lines with the exception of the MCF-7 cell line, which must be considered CAR-deficient. Moreover, combining estradiol or tamoxifen with TSA did not result in an additive enhancement of CAR expression. This argues against there being two distinct mechanisms of action for up-regulating CAR by means of the investigated drugs and suggests that histone acetylation is influenced to a lesser extent by estradiol and tamoxifen than by the hyperacetylating agent TSA. Our data are consistent with the findings of Sun et al. [20], who reported that estradiol increased the level of acetylated histones by reducing the rate of histone deacetylation in ER-positive but not negative breast cancer cell lines. The hypothesis that activated ER

alters the balance of histone acetyltransferases and HDACs at the nuclear matrix as well as the dynamics of acetylation of histones associated with transcriptionally active and competent chromatin, could reliably explain the herein described association between ER positivity and high hCAR expression in breast cancers and the inducibility of hCAR expression in receptor-positive cell lines through ER activation.

At first glance, the fact that tamoxifen and also TSA induce expression of hCAR could represent a suitable approach ameliorating the transduction efficiency of adenoviral vectors in gene therapeutic strategies in ER-positive breast cancers. However, an additional important finding of our investigations is that the soluble isoforms of CAR, which are known to significantly interfere with cell entry of adenoviral particles [21], were also considerably up-regulated by tamoxifen or TSA treatment and might diminish the favorable effect of enhanced membranous CAR expression on therapeutic gene delivery.

In conclusion, we here describe an unknown association between estrogen receptivity and the expression of membranous CAR in breast cancer. As a proof of principle, inducibility of membranous CAR and its soluble isoforms by estradiol and tamoxifen was demonstrated in hormone receptor-positive breast cancer cell lines. As a consequence of these findings we suggest that the co-expression of ER and CAR in breast cancers is not only related to a more highly differentiated tumor phenotype, but also founded on a functional basis.

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