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Soluble isoforms but not the transmembrane form of coxsackie-adenovirus receptor are of clinical relevance in epithelial ovarian cancer

Reimer D, Steppan I, Wiedemair A, Concin N, Hofstetter G, Marth C, Müller-Holzner E and Zeimet AG*

Department of Obstetrics and Gynecology, Innsbruck Medical University, Innsbruck, Austria

The coxsackie-adenovirus receptor (hCAR) has been extensively studied in context of adenoviral-based gene therapy for cancer. However, there is strong evidence that besides its decisive role in coxsackie and adenovirus cell-entry, hCAR is a component of epithelial tight junctions and involved in cell-cell adhesions in normal and cancer cells. Furthermore, this adhesion molecule behaves like a cell surface receptor endowed with tumor suppressive properties *via* signal transduction. Moreover, 3 truncated soluble isoforms of hCAR were recently identified. We investigated the quantitative expression of all known CAR isoforms in a training set of 140 ovarian cancer samples and 21 controls by RT-PCR. The expression levels of the various isoforms were compared with clinicopathologic parameters and their prognostic significance was assessed. Expression levels of all CAR isoforms were elevated in ovarian carcinomas as compared with those of non-malignant controls. mRNA-expression correlated with protein levels. Moreover, expression of the soluble isoforms CAR 3/7 and CAR 4/7 but not that of hCAR was significantly increased in advanced ovarian cancer as revealed by a highly significant correlation with FIGO stage and residual disease > 2 cm in diameter after debulking surgery. High expression of CAR 3/7 and 4/7 was shown to be of independent prognostic relevance for progression-free (CAR 4/7) and overall survival (CAR 3/7 and CAR 4/7). In conclusion, soluble CAR isoforms 3/7 and 4/7 may play a pivotal role in ovarian cancer biology, possibly by counteracting migration- and growth-inhibitory properties of the membranous hCAR and thus favoring cancer cell dissemination throughout the peritoneal cavity.
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Key words: ovarian cancer; coxsackie-adenovirus receptor; soluble isoforms; prognosis

Epithelial ovarian cancer is the leading cause of death from gynecologic malignancies. Because the majority of patients present with advanced disease at diagnosis and despite a high objective response rate to initial platinum-based chemotherapy, five-year overall survival (OS) remains poor with less than 40% survivors[1] because of rapid development of resistance to chemo-therapy. Therefore, great effort has been undertaken in developing alternative therapy strategies, such as gene therapy *via* adenovirus vector systems.[2–6] As trans-gene delivery and thus therapy efficacy correlates with susceptibility to transduction,[7–10] expression and function of the full-length form of the membranous human coxsackie adenovirus receptor (hCAR), which is the primary attachment protein for adenovirus entry into the cell, has been extensively studied during recent years. CAR belongs to the immunoglobulin superfamily.[11] The 46 kDa glycoprotein is composed of 2 extracellular immunoglobulin domains (D1 and D2), an α-helical transmembrane domain (TMD) and a cytosolic region (CR) (Fig. 1).[12] The human CAR gene is located on chromosome 21q11.2 consisting of at least 8 exons distributed over an area of 54 kb.[13] Exon I encodes the *N*-terminal leading sequence, whereas D1 is encoded by Exons II and III, D2 by Exons IV and V. The first part of Exon VI constitutes the TMD, and the cytosolic domain of CAR is coded by the last part of Exon VI and Exon VII (and VIII). Because of alternative RNA splicing between Exons VII and VIII, 2 membranous hCAR isoforms (hCAR1 and hCAR2) exist containing an intact extracellular domain and TMD with modifications in the cytoplasmatic domain.[14,15] In a recent study the 2 transmembranous isoforms were shown to be co-expressed in most analyzed murine epithelial cells.[16] Exon-skipping of Exons III–VI in CAR 2/7 (γ), Exons IV–VI in CAR 3/7 and Exons V–VI in CAR 4/7 (β) results in at least 3 other CAR splice variants, which contain the leading sequence encoded by Exon I, different portions of the immunoglobulin domains, but lack the TMD (Fig. 1).[17,18] These extracellular secreted, soluble CAR isoforms can inhibit coxsackievirus infection, probably *via* binding to the extracellular portion of hCAR and the coxsackie virus B3 (CVB3) capsid, indicating their possible role in host defense against viral infection.[17] Moreover, in malignant pleural effusions a soluble CAR variant was shown to interfere with adenoviral gene transfer in an inhibiting manner.[19] However, the role of hCAR and its soluble isoforms in the pathophysiology of malignant disease is not fully understood. There is growing evidence that hCAR is an important component of interepithelial tight junctions (TJs), suggesting its possible role in cell adhesion and migration. Within the TJs hCAR has been shown to colocalize with known TJ-associated proteins such as zonula occludens-1 (ZO-1),[20] the multi-PDZ-containing proteins MUPP-1,[21] MAGI-1b, PICK1 and PSD-95[22] and LNX and LNX-2.[23,24] A possible functional modulation of cell adhesion is mediated through peptide motifs, which are known to interact with PDZ protein domains, located on the hydrophobic C-terminus of hCAR. Indeed, hCAR was shown to inhibit cancer cell migration in hCAR transfected ovarian and cervix cancer cell lines.[25] Moreover, several studies performed in human tumor cell lines, including prostate cancer, bladder cancer and glioblastome multiforme, indicate reduced hCAR expression during tumor progression.[26–29] In bladder cancer cell lines hCAR was shown to be involved in modulating cell cycle regulators, such as $p21^{CIP}$ and Rb, suggesting a possible role of hCAR as a tumor suppressor.[27] However, in breast cancer hCAR was shown to be elevated as compared with background tissue, and increased levels of hCAR transcript were associated with tumor progression, higher grade of malignancy, nodal involvement and poor long-term survival.[30] The role of hCAR in tumorigenesis seems to differ in various tumor entities and remains a major matter of debate. Furthermore, to our knowledge no data are available on the expression and clinical impact of hCAR and especially of its soluble isoforms in ovarian cancer. We here report for the first time on the expression of hCAR and its soluble variants in a large training set of 140 ovarian cancer samples. In the light of possible cross-talk between membranous and soluble isoforms, the relevance of these adhesion molecules in tumor dissemination and in predicting disease-free and OS in ovarian cancer was studied.

Abbreviations: hCAR, human coxsackie-adenovirus receptor (full length form); hCAR1, membranous isoform-1 of hCAR; hCAR2, membranous isoform-2 of hCAR; CF, cytosolic fraction; DFS, disease free survival; MF, membrane fraction; OS, overall survival; Rb, retinoblastoma tumor suppressor protein; TBP, TATA box binding protein; TJs, tight junctions.

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***Correspondence to:** Department of Obstetrics and Gynecology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria. Fax: +43-512-504-23055. E-mail: alain.zeimet@uibk.ac.at

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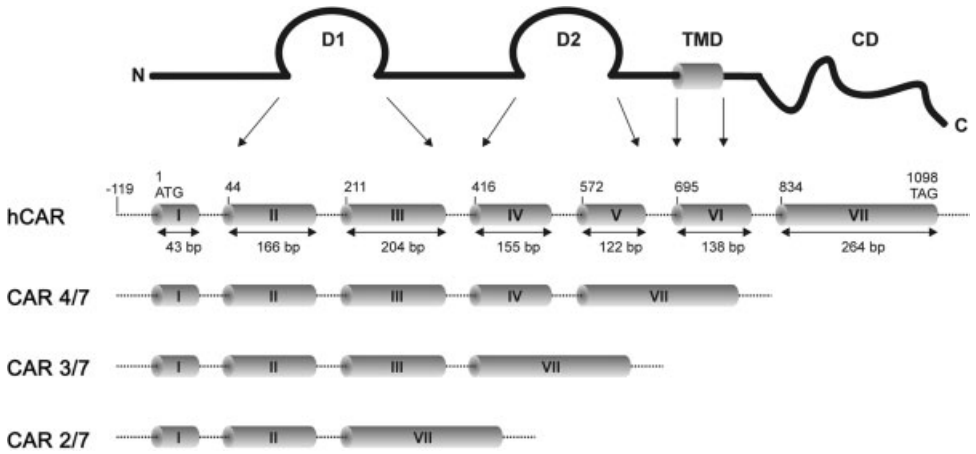

FIGURE 1 – Schematic presentation of the hCAR protein and its soluble isoforms CAR 4/7, CAR 3/7 and CAR 2/7. The 2 extracellular immunoglobulin domains (D1 and D2) are encoded by exons II and III (D1) and by Exons IV and V (D2). The TMD is encoded by the first part of Exon VI, thus not existent in the soluble splice variants. CD, cytoplasmatic domain.

Material and methods

Patients

Tissue samples from patients with invasive, epithelial ovarian cancer were collected during primary debulking surgery at the Department of Obstetrics and Gynecology in Innsbruck between 1998 and 2004 ($n = 140$). Our patient cohort represented two third of the total of patients ($n = 232$) who underwent primary surgery for ovarian cancer at the department during that period. Tumors with borderline malignancy were excluded. Ovarian tissue samples obtained from patients during surgery for other than inflammatory or malignant conditions served as control ($n = 21$). All the 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 either used for subsequent RNA extraction or stored at −80°C.

Cell lines

Various established ovarian cancer cell lines, HTB-77, OVCAR-3, SKOV-6, A2780, AG6000 (a dCK-deficient A2780 cell line kindly provided by Prof. Peters, Department of Molecular Biosciences, Swedish University of Agricultural Sciences, Uppsala, Sweden) and the CAR-deficient bladder cancer cell line T24 were cultured under the same conditions published recently.[31] Human peritoneal mesothelial cells (HPMC) and ovarian surface epithelial cells (OSE) served as control and were obtained from patients during surgery for other than inflammatory or malignant disease.

Immunoblotting

Based on fresh frozen tissue availability immunoblot analyses were performed in a subset of 25 ovarian cancer samples and 5 healthy controls. Tissue samples were homogenized in a 10 mM Tris HCl (pH 7.4) buffer containing 5 mM EDTA-Tris, 5 μg/ml Aprotinin, 4 μg/ml Leupeptin, 1 μM Pepstatin A, 0.1 mM PMSF and centrifuged at 1000 × *g* for 15 min to remove insoluble debris. Membrane protein fraction (MF) was separated from the cytosolic fraction (CF) by centrifugation at 100,000 × *g* for 20 min. Equal amounts of protein (30 μg) were separated on 12% SDS-PAGE under reducing conditions, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated with the monoclonal mouse anti-CAR RmcB (0.5–1.5 μg/ml) (Millipore, Billerica, MA), washed and then incubated with goat anti-mouse IgG (1:60,000) (Sigma-Aldrich). HRP activity was detected using enhanced chemiluminescence (ECL plus, Amersham Biosciences, Vienna, Austria). The intensity of protein expression determined in the immunoblot analyses was correlated with means of mRNA expression levels. Protein band intensities were defined as the sum of pixels within the area of the band limited by a preformed rectangular area after subtraction of the background pixels.

RNA extraction and RT reaction

Total RNA was isolated from patient samples using the guanidium thiocyanate-phenol-chloroform method according to the manufacturer's protocol (RNAgents® 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.[31]

Primers and probes

Specific primers and probes for the coxsackie adenovirus receptor (hCAR), its soluble splice-variants CAR 2/7, CAR 3/7 and CAR 4/7 and for the TATA box-binding protein (TBP; a component of the DNA-binding protein complex TFIID as an endogenous RNA control) were determined with the assistance of 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 2 Exons. The different splice variants were distinguished by placing the forward primer between Exons II and VII for CAR 2/7, Exons III and VII for CAR 3/7, Exons IV and VII for CAR 4/7 and between Exons VI and VII for hCAR. The reverse primer (CAR ExVII AS) and the probe (CAR ExVII Taq) used for all splice variants were placed in Exon VII. In addition, hCAR1 and hCAR2 specific primer sets were designed in order to evaluate a possible difference in expression between the 2 C-terminal spliced membranous hCAR isoforms. Sequences of primers and probes are given in Table I.

Real-time PCR amplification

RT-PCR was performed using an ABI Prism 7900 Detection System (Applied Biosystems, Foster City, CA) in a total volume of 25 μl reaction mixture containing 5 μl of each appropriately diluted RT sample (standard curve points and test samples), 12.5 μl 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 sec and 65°C for 1 min. Real-time PCR efficacies were acquired by amplifying serially diluted cDNA isolated from the ovarian cancer cell line HTB-77. PCR assays were conducted in triplicate and the mean value was used for calculation. Only PCR experiments with a detection efficiency over 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

TABLE I – PRIMERS AND PROBES OF REAL-TIME PCR

Isoform	Exon	Oligonucleotide	Sequence
CAR 2/7	2/7	Forward primer	5′-GGT GGA TCA AGT ggg aag atg t-3′
CAR 3/7	3/7	Forward primer	5′-TCT GGT AGT TCT TGG GAA GAT GTG-3′
CAR 4/7	4/7	Forward primer	5′-CAT GGT TAG CAG GGA AGA TGT G-3′
hCAR	6/7	Forward primer	5′-AGT TCA TCA CGA TAT CAG GGA AGA T-3′
CAR_Ex7_AS	7	Reverse primer	5′-TGA ATG ATT ACT GCC GAT GTA GC-3′
CAR_Ex7_Taq	7	Probe	5′-FAM-CAC CTC CAA AGA GCC GTA CGT CCA CTG-TAMRA-3′
hCAR1	7	Reverse primer	5′-CGC ACC CAT TCG ACT TAG ATT AG -3′
hCAR2	7	Reverse primer	5′-TCC ATC AGT CTT GTA AGG GTA CTT GA -3′
hCAR_Ex7_Taq	6/7	Probe	5′-FAM-CAG AGT CCG ACT CTC CCA CCT GCT AAG -TAMRA-3′
hCAR_Ex6_S	6	Forward primer	5′-AGT GAA GAC TTT GAA CGC ACT CC -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. hCAR1 and hCAR2 isoforms were discriminated by specific exon 7 located reverse primers.

TABLE II – CLINICOPATHOLOGIC CHARACTERISTICS OF THE TRAINING SET

	n	Median (Q_1-Q_3)
Age (y)	140	62.6 (52.06–73.27)
Median DFS (m)		23.04 (0.1–58.44)
Median OS (m)		42.96 (5.10–80.82)
	n	%
Death during follow-up		
Yes	82	(58.6)
No	58	(41.4)
Relapse during follow-up		
Relapse	95	(67.9)
No relapse	45	(32.1)
FIGO stage		
I	25	(17.9)
II	12	(8.6)
III	80	(57.1)
IV	23	(16.4)
Histologic type		
Serous	68	(48.6)
Mucinous	40	(28.6)
Endometriod	22	(15.7)
Others	10	(7.1)
Histopathologic grading[1]		
1	5	(3.6)
2	73	(52.1)
3	60	(42.9)
Residual disease[2]		
No residual disease	48	(34.3)
Residual disease < 2cm	33	(23.6)
Residual disease > 2cm	58	(41.4)
Operative therapy		
Yes	138	(98.6)
No	2	(1.4)
Chemotherapy		
Yes	127	(90.7)
No	13	(9.3)

[1]The histopathologic grading of one patient was not available.–[2]Residual disease after debulking surgery was not available in one patient. Abbreviations: y, years; m, month; FIGO, Fédération Internationale de Gynécologie et d′Obstétrique.

in patient samples were normalized to the TATA box binding protein (TBP).

Clinicopathologic characteristics

All patients ($n = 140$) were monitored within the outpatient follow-up program of the Department of Obstetrics and Gynecology, Innsbruck, and the median observation period of included patients was 43 (5–81) months. Clinicopathologic characteristics are summarized in Table II. Primary debulking surgery was performed in all but 2 patients, who received carboplatin-based chemotherapy due to impaired performance status. With the exception of 13 patients who presented with low grade tumors in FIGO stages I a and b, all patients received 6 cycles of a platinum-based chemotherapy (90.7%). Forty-five patients (32.1%) did not show recurrent disease and 82 of 140 patients (58.6%) died during follow-up.

Statistical analysis

Differences in expression level of CAR transcripts in cell culture experiments and between expression levels of hCAR1 and hCAR2 in the subset analysis were assessed with the Student's t-Test, between normal and malignant tissues with the Mann-Whitney-U-Test. Because CAR expression showed neither a clear negative value, nor a biphasic distribution, and no clinically relevant cut-off levels are available for CAR expression levels in ovarian cancer, we had to determine the optimal cut-off level of all CAR splice variants in our set of 140 patients. For this purpose each percentile from 10th to 75th was calculated for CAR expression and used as the cut-off point. At each of these cut-off values, the data were dichotomized into 2 groups of low and high expression and difference in OS was assessed with the log-rank test. The greatest difference between the 2 groups was defined as the optimal clinical cut-off value.

Furthermore, patients were dichotomized according to low or high CAR expression levels and intergroup differences concerning clinicopathologic characteristics were evaluated with the Mann-Whitney-U-Test for quantitative data. Survival analyses were conducted for DFS (disease-free survival) and OS (overall survival) with the Kaplan-Meier method and differences between groups were determined with the log-rank test. To assess the predictive value of CAR expression the Cox proportional hazard model with a stepwise backward method was used with adjustment for confounding variables. The final model included age at diagnosis, histopathologic grading, histologic subtype, FIGO stage, residual disease after primary debulking, and the levels of CAR expression. The relative risk for recurrence or death was expressed as ratio with 95% CI. Statistical significance was defined as $p < 0.05$. SPSS for Windows 12.0 software (SPSS, Chicago, IL) was used for all analyses.

Results

Expression of hCAR and its soluble isoforms in cancer cell lines

To evaluate our primer sets we used RT-PCR to assess quantitative expression levels of full-length coxsackie-adenovirus receptor (hCAR) and its soluble splice variants CAR 2/7, CAR 3/7 and CAR 4/7 in various cancer cell lines. Since the hCAR sense primer was placed between Exon VI and Exon VII both presently known transmembranous hCAR isoforms (hCAR1 and hCAR2) were recognized by our primer set. The expression levels of CAR variants in various cancer cell lines are shown in Table III.

Interestingly, all CAR variants were expressed to a higher extent in cultured HPMC and OSE cells than in most of the investigated tumor cell lines (Table III). In HPMC, most prominent expression levels were observed for hCAR and soluble CAR 2/7 (9.57 ± 0.99 and 10.69 ± 1.05, respectively). All CAR isoforms were expressed to a lesser extent in OSE cells than in HPMC. Again, highest tran-

TABLE III – REAL-TIME PCR OF CANCER CELL LINES

	hCAR	CAR 2/7	CAR 3/7	CAR 4/7
OVCAR-3	8.99 (±1.04)	*12.96 (±1.43)	*7.84 (±0.79)	*5.14 (±0.58)
SKOV-6	**1.78 (±0.26)	**2.52 (±0.18)	**1.67 (±0.09)	**1.47 (±0.08)
A2780	**0.26 (±0.03)	**0.26 (±0.05)	**0.19 (±0.02)	**0.22 (±0.01)
AG6000	**1.11 (±0.15)	**0.92 (±0.07)	**0.65 (±0.07)	**0.62 (±0.07)
T24	**0.01 (±0.01)	**0.01 (±0.01)	**0.01 (±0.01)	**0.01 (±0.01)
OSE	5.31 (±0.56)	5.96 (±0.52)	3.59 (±0.60)	3.68 (±0.29)
HPMC	9.57 (±0.99)	10.69 (±1.05)	6.18 (±0.65)	6.87 (±0.70)

Values are given as mean (±SD) of 3 independently performed experiments each done in triplicate. Transcript levels are expressed as ng *to*tal RNA. CAR was normalized to ng total RNA of TBP. Differences in expression levels between tumor cell lines and HPMC were evaluated with Student's *t*-test; statistical significance is indicated by asterisk: $^{*}p < 0.05$, $p < 0.01$. HPMC, human peritoneal mesothelial cells; OSE, ovarian surface epithelial cells.

TABLE IV – REAL-TIME PCR OF THE TRAINING SET (MEDIAN AND INTERQUARTILE RANGE)

	Normal ($n = 21$)	Ovarian cancer ($n = 140$)	p value[1]
CAR 2/7	0.1 (0.00–0.24)	3.09 (1.83–4.34)	0.0001
CAR 3/7	0.11 (0.03–0.19)	2.97 (1.74–4.21)	0.0001
CAR 4/7	0.18 (0.11–0.24)	3.12 (1.68–4.56)	0.0001
hCAR	0.19 (0.08–0.3)	3.27 (2.06–4.47)	0.0001

Transcript levels are expressed as ng total RNA. CAR was normalized to ng total RNA of TBP.

[1]Differences between the two groups were evaluated with the Mann-Whitney-U-Test.

script levels were determined for hCAR and CAR 2/7 (5.31 ± 0.56 and 5.96 ± 0.52, respectively). In ovarian cancer cell lines expression patterns of CAR isoforms were heterogeneous, ranging from very low transcript levels determined in HTB-77, SKOV-6, A2780 and AG6000 to high expression in the OVCAR-3 cell line. Highest expression levels in OVCAR-3 cells were detected for hCAR and CAR 2/7 (8.99 ± 1.04 and 12.96 ± 1.43, respectively). As expected, none of the CAR isoforms were detectable in the CAR-deficient bladder cancer cell line T24 (Table III).

In vivo *expression of CAR receptor and its soluble splice variants in ovarian cancer*

Expression levels of hCAR and its soluble splice variants CAR 2/7, CAR 3/7 and CAR 4/7 were assessed in 140 ovarian cancer samples and 21 controls (Table IV). In contrast to the data obtained in cultured HPMC and OSE, hCAR and its 3 soluble splice variants were expressed at very low levels in normal ovarian tissue, whereas up to 32-fold higher expression levels (CAR 2/7) were observed in the ovarian cancer samples. Most prominent expression levels were detected for hCAR (median value 3.27 *vs.* 0.19 in controls), CAR variant 4/7 (median value of 3.12 *vs.* 0.18 in controls) and CAR variant 2/7 (median value of 3.09 *vs.* 0.1 in controls). CAR 3/7 expression was enhanced to a lesser extent (Table IV).

In a subset of 25 ovarian cancer samples and 10 controls membranous hCAR1 and hCAR2 isoforms were discriminated using hCAR1- and hCAR2-specific primer sets. Expression levels of hCAR1 and hCAR2 transcript levels highly correlated with that observed for hCAR expression ($\rho = 0.897$, $p = 0.0001$ and $\rho = 0.708$, $p = 0.0001$, respectively). Furthermore, hCAR1 expression was significantly higher as compared with hCAR2 ($p = 0.0001$). Whereas, transcript levels of hCAR1 and hCAR2 were near the detection limit in controls, in ovarian cancer samples both membranous splice variants revealed to be elevated 10-fold and 7-fold, respectively (hCAR1: median value of 3.36 *vs.* 0.34 in controls, $p = 0.0001$; hCAR2: median value of 2.36 *vs.* 0.33 in controls, $p = 0.0001$).

Although no significant correlation of expression levels of all soluble splice variants was detected when groups were subdivided according to histologic subtype and histopathologic grading, expression levels of the soluble CAR 3/7 and CAR 4/7 were elevated in patients with FIGO stages III and IV tumors ($p = 0.015$ and $p = 0.026$, respectively), as compared with carcinomas diagnosed in FIGO Stages I and II. Expression of CAR variant 2/7 and hCAR was equally distributed among the various FIGO stages. Furthermore, CAR 3/7 and CAR 4/7 transcript levels were found to be significantly higher in patients with residual disease >2 cm in diameter after primary debulking surgery as compared with optimally debulked patients (no residual disease or residual disease <2 cm) ($p = 0.019$ and $p = 0.034$, respectively). Again, CAR 2/7 and hCAR expression was equally distributed between groups with residual disease >2 cm in diameter and patients with optimal debulking surgery.

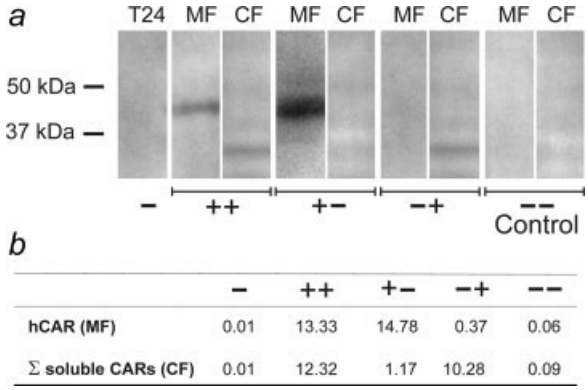

FIGURE 2 – Protein expression of the 46 kDa band representing hCAR in the membrane fraction (MF) and the 31 kDa band representing soluble CAR isoforms in the cytosolic fraction (CF) in ovarian cancer determined by immunoblot analyses (*a*). Three patients representing different mRNA expression patterns of hCAR and soluble CAR isoforms are shown. No protein was detected in the CAR deficient cell line T24 and expression levels were near the detection limit in control sample (Control). CAR mRNA transcript levels (ng total RNA) of the tissue samples shown in (*a*) (normalized to TBP in ng total RNA) are shown (*b*). Values are given as mean of triplicates. hCAR, human coxsackie adenovirus receptor; Σ soluble CARs, soluble CAR receptors considered together.

Interestingly, expression levels of all soluble CAR isoforms were significantly elevated in carcinomas with proven mutations in the *p53* gene, as compared with those with wild-type p53 (CAR 2/7: $p = 0.045$; CAR 3/7: $p = 0.039$; CAR 4/7: $p = 0.007$), whereas hCAR expression was independent of p53 status. Furthermore, E2F1 transcript levels were found to correlate with the expression levels of hCAR ($\rho = 0.205$; $p = 0.019$) and with soluble CAR isoforms (CAR 2/7: $\rho = 0.237$, $p = 0.007$; CAR 3/7: $\rho = 0.292$, $p = 0.001$; CAR 4/7: $\rho = 0.223$, $p = 0.011$).

Expression of CAR protein in ovarian cancer samples

In order to exclude relevant post-translational modifications in CAR protein biosynthesis mRNA expression profiles of CAR isoforms were compared with protein expression in 25 ovarian cancer

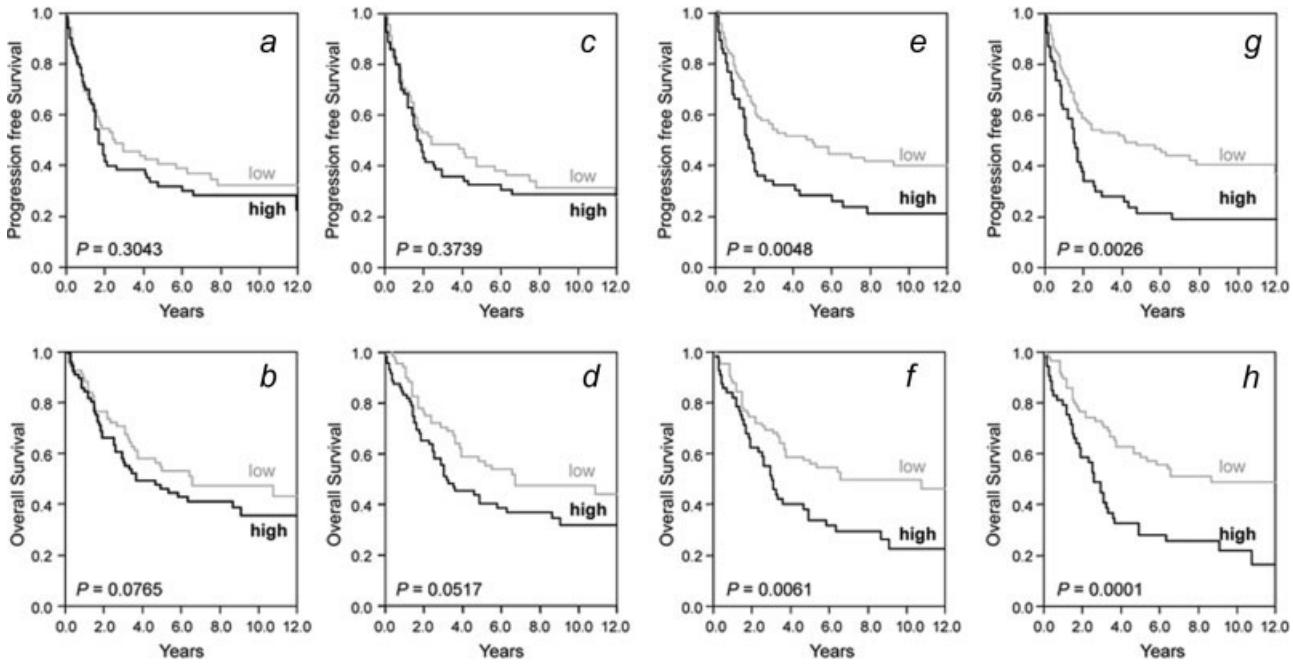

FIGURE 3 – Univariate survival analyses for disease-free survival (DFS) and overall survival (OS) for patients with ovarian cancer according hCAR (*a*,*b*), CAR 2/7 (*c*,*d*), CAR 3/7 (*e*,*f*) and CAR 4/7 (*g*,*h*) expression levels. High expression levels (High) of CAR 3/7 and CAR 4/7 were significantly associated with poor DFS and OS. Differences in expression were assessed by using the log-rank test.

samples and 5 control tissues by the use of immunoblot analyses. Comparison of hCAR transcript levels with the intensity of the 46 kDa band representing the membranous CAR protein showed a significant correlation ($\rho = 0.457$, $p = 0.034$). Although, the various soluble CAR isoforms were not distinguishable by immunoblot analyses due to their minor differences in molecular weight, the specific bands detected exclusively in CF at 31 kDa were significantly correlated with the mRNA transcript levels of the 3 soluble isoforms when considered together ($\rho = 0.424$, $p = 0.046$). Membrane and CFs of 3 patients and 1 healthy control sample representing different mRNA transcript profiles are shown as *pars-pro-toto* examples in Figure 2*a*. CAR immunoreactivity was near the detection limit in the healthy control samples and non-visible in the CAR deficient T24 cell line, which served as a negative control.

Prognostic relevance of CAR expression in ovarian cancer

To validate our data set, univariate survival analyses of well-defined prognostic parameters were performed. As expected, DFS and OS were significantly influenced by FIGO state ($p = 0.0003$ and $p = 0.00001$, respectively), by residual disease ($p = 0.00001$ and $p = 0.00001$, respectively), and by histologic grade of differentiation ($p = 0.001$ and $p = 0.0002$, respectively). Age at diagnosis over 62 years (52.06–73.27) predicted poor OS, but not DFS ($p = 0.001$ and $p = 0.07$, respectively). The histologic subtype did not influence DFS or OS. Since no data are available on the clinical relevance of the expression of hCAR and its soluble isoforms in ovarian cancer, the optimal cut-off value reflecting prognostic relevance had to be determined in the described training set of patients. For this purpose, survival curves applying cut-off levels between the 10th and 75th percentiles were calculated. Highest level of significance was reached at a cut-off corresponding to the 50th percentile (cut-off value: 3.09) for CAR 2/7, the 60th percentile (cut-off value: 3.54) for CAR 3/7 and the 62nd percentile for CAR 4/7 (cut-off value 3.87). No statistical significant threshold level was found for hCAR.

In univariate survival analyses patients with high expression levels of CAR 3/7 and CAR 4/7 showed significantly shorter DFS and OS (Figs. 3*e*–3*h*). Median time to progression for patients with high and low CAR 3/7 transcript levels was 17 *versus* 35 months, respectively ($P = 0.048$). Median OS was 36 *versus* 79 months, respectively ($P = 0.006$). Concerning CAR 4/7 expression levels, median time to progression was 18 *versus* 46 months, respectively ($p = 0.003$), median OS for patients with high and low transcript levels was 31 *versus* 104 months, respectively ($p = 0.0001$). High CAR 2/7 expression was associated with poor OS, but did not reach statistical significance for DFS (Figs. 3*c* and 3*d*). No statistical significant difference was observed for hCAR expression (Figs. 3*a* and 3*b*).

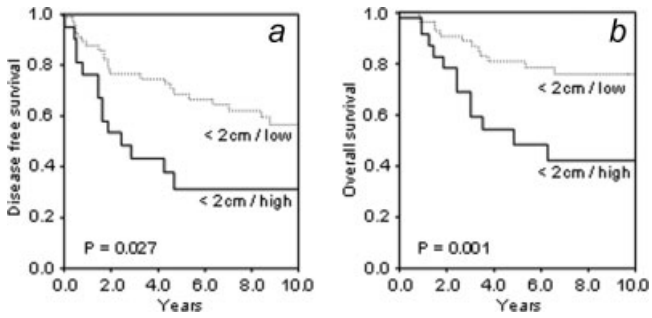

FIGURE 4 – Univariate survival analyses for disease-free survival (*a*) and overall survival (*b*) for patients with ovarian cancer according CAR 4/7 expression levels in optimally debulked patients (tumor residual <2 cm). Differences in expression were assessed by using the log-rank test.

When patients were stratified according to residual disease over 2 cm in diameter after debulking surgery and optimally debulked patients (<2 cm and no residual disease), CAR 4/7 expression did not significantly influence DFS or OS in the group with residual disease >2 cm in diameter (not shown). Interestingly, high expression levels of CAR 4/7 significantly deteriorated DFS and OS in the subgroup of the optimally debulked patients (Figs. 4*a* and 4*b*). In optimally debulked patients with tumors exhibiting high CAR 4/7 transcript levels median time to progression was 35 months

TABLE V – SOLUBLE CAR SPLICE VARIANTS WITH INDEPENDENT PROGNOSTIC SIGNIFICANCE FOR DFS AND OS

Variable	DFS			OS		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Age at diagnosis						
<median	1.000			1.000		
>median	2.062	(1.337–3.180)	0.001	3.116	(1.931–5.030)	0.0001
Residual Disease						
Optimally debulked	1.000			1.000		
Tumor residual > 2 cm	2.819	(2.082–3.815)	0.0001	3.089	(2.205–4.328)	0.0001
CAR 3/7						
Low	1.000			1.000		
High	1.241	(0.813–1.895)	0.317	1.683	(1.061–2.672)	0.027
CAR 4/7						
Low	1.000			1.000		
High	1.554	(1.020–2.370)	0.040	2.214	(1.405–3.490)	0.001

Cox regression hazard model with the stepwise backward method. Only values with statistical significance are shown. DFS, disease free survival; OS, overall survival; CI, confidence interval.

and median OS 59 months, whereas neither median time to progression nor median OS was reached in tumors with low CAR 4/7 expression ($p = 0.0001$ and $p = 0.0001$, respectively) (Figs. 4*a* and 4*b*). Similar results were obtained when CAR 3/7 expression levels when applied at the determined cut-off value of 3.54 (not shown).

In the multivariate Cox regression with a stepwise backward method expression of the soluble splice variant CAR 4/7 was found to be an independent prognosticator for DFS and OS and CAR 3/7 was shown to be of independent prognostic relevance for OS but not for DFS. Of the other clinicopathologic parameters included in the model, age at diagnosis and residual disease retained independent prognostic significance for both DFS and OS (Table V).

Discussion

Over the past decade great efforts have been made to optimize gene therapy *via* adenoviral-vector systems as an alternative strategy in the treatment of various tumor entities, including ovarian cancer.[3,6] As therapy efficacy was shown to correlate strongly with the ability of the virus to enter into cancer cells, expression and function of the coxsackie-adenovirus-receptor (hCAR), which is the primary receptor of group C adenoviruses, were studied extensively. hCAR was shown to be differently expressed in various tumor entities, and at least in ovarian cancer immunohistochemical investigations revealed a marked distributional heterogeneity with regard to focal positivity and negativity in the same tumor.[32] The herein presented approach in determining hCAR and its soluble isoforms by RT-PCR showed that the known CAR isoforms were considerably expressed in ovarian cancer tissue and respective transcripts were found to be significantly higher in cancer samples than in normal ovarian tissue samples, obtained from patients during surgery for other than malignant or inflammatory disease. However, this was not true when highly CAR-positive normal HPMC or OSE cells were regarded as normal reference. The discrepancy between normal ovarian tissue and cultured HPMC and OSE cells concerning the expression of CAR isoforms is most probably due to contamination of the tissue samples with ovarian stromal tissue, characterized by very low CAR expression. On the other hand we can not completely rule out that these molecules are differently expressed in three-dimensional tissue texture as compared with low-anchorage *in vitro* conditions.

The low expression of CAR isoforms found in most of the investigated ovarian cancer cell lines is in line with data published by other groups.[25,33] As OVCAR-3 cells, however, exhibit high CAR transcript levels comparable with those detected in HPMC, a heterogeneity of CAR expression among ovarian cancer cell lines must be postulated in agreement with the results published by Wang *et al*.[33]

In addition to its key role in adenovirus cell entry, several authors have also implicated hCAR in molecular processes of tumor biology and progression, but its exact role in tumor cell proliferation and dissemination remains largely unclear. This is especially true when possible functional cross-talk between hCAR and its recently discovered, soluble splice variants is taken into consideration.

Moreover, the functional impact of C-terminal splicing of hCAR resulting in 2 membranous splice variants hCAR1 and hCAR2 remains to be eludicated. In murine tissues both isoforms were shown to be co-expressed in most epithelial cell-cell contacts.[16] However, no data concerning functional differences of hCAR1 and hCAR2 are currently available. In ovarian cancer both membranous isoforms are expressed to a higher extent compared to healthy control tissue, where expression is near the detection limit. Expression of hCAR2 was shown to be inferior to hCAR1 and both membranous isoforms together represented expression determined with the hCAR primer set. Moreover, expression patterns of both isoforms were highly correlated with hCAR expression in the subset analysis and therefore at least in ovarian cancer all membranous isoforms seem to be comparable with regard to their clinical impact.

Most notably, hCAR was shown to be associated with (TJs) in mammalian epithelial cells. Using confocal microscopy and thin-section electron microscopy, hCAR was found to be located at the apical pole of the lateral membrane of polarized epithelial cells, where it colocalizes with ZO-1, the central structural protein of TJs.[20] Moreover, interaction of various PDZ-scaffolding proteins including MUPP-1,[21] MAGI-1b, PICK1 and PSD-95[22] and LNX and LNX-2[23,24] with C-terminal PDZ-motifs was reported. However, the functional aspect of these interactions remains to be elucidated. Raschperger *et al*. reported that CAR expression correlates with the maturity of TJs and is inversely associated with permeability of TJs in mice.[16] In addition, enrichment of hCAR has also been observed by immunofluorescence visualization at cell-cell contact sites in human ovarian and cervical cancer cells.[34] These findings suggest a pivotal role for hCAR in cell-cell adhesion and consequently in cell migration in cancer. Indeed, hCAR re-expression resulted in formation of cell-cell contacts and aggregation in cell clusters in otherwise singularly growing A2780 ovarian cancer cells, and cell migration was strongly reduced in spread-off assays.[25] Furthermore, transfection of hCAR into the CAR-deficient bladder cancer cell line T24 not only increased adenovirus transduction efficacy, but also inhibited its *in vitro* growth.[27,28] Similar *in vitro* results were reported for malignant glioma cells and the ovarian cancer cell line A2780.[25,29] In transitional cell cancer (TCC) cells, hCAR-induced growth inhibition was linked to cell cycle arrest with accumulation of p21 and hypophosphorylated Rb protein, and the intracellular domain of hCAR was identified as being critical for inducing its growth inhibitory activity.[27]

These results show that hCAR obviously behaves like a classic membrane receptor that conveys its signals into the nucleus, resulting in suppression of cell proliferation. The postulated tumor suppressive function of hCAR is further supported by the down-regulation of hCAR in the highly tumorigenic prostate cell line PC-3[26] and by the inverse relationship between hCAR expression in human bladder cancer tissue and tumor stage and histopathologic grade of differentiation.[27,28] Similar results regarding tumor grade and immunohistochemical hCAR staining were also reported by Rauen *et al*. in prostate cancer tissue. However, these authors unexpectedly found significantly higher hCAR expression in metastatic lesions than in the corresponding primary tumors.[35] Furthermore, in human breast cancer hCAR was reported to be elevated as compared to background tissue, increasing with tumor *grade* and metastatic disease.[30]

In a training set of 140 ovarian cancer patients we found that high expression of the soluble isoforms CAR 3/7 and CAR 4/7 but not of hCAR is associated with advanced disease as revealed by highly significant correlations with FIGO stage and residual disease after primary debulking surgery. Furthermore, both mentioned soluble isoforms have been shown to be of independent prognostic relevance for DFS (CAR 4/7) and OS (CAR 3/7 and CAR 4/7), and high expression levels of CAR 4/7 or CAR 3/7 deteriorate clinical outcome even in optimally debulked patients as shown in univariate survival analysis.

Although, herein revealed prognostic relevance of soluble CAR variants 3/7 and 4/7 could be interpreted as an epiphenomenon related to cancer progression, a causative link between overexpression of soluble CAR isoforms and aggressive tumor behavior can not be ruled out. Our findings may suggest that in ovarian cancer biology soluble isoforms of CAR display an opposite function to that postulated for transmembrane hCAR. As Dorner *et al*. found that soluble CAR proteins are able to interact with the bacterially expressed extracellular domain of hCAR[17] and the extracellular domain was shown to be critical in homodimer formation of hCAR,[36] it is tempting to speculate that CAR 4/7 and CAR 3/7 antagonize the tumor suppressive functions of hCAR *via* direct interaction with its extracellular domain. Insertion of soluble CAR variants into the protein network of TJs may destabilize cell-cell adhesion and significantly contribute to the detachment of single cancer cells from the primary tumor. In ovarian cancer, which usually remains confined to the peritoneal cavity throughout its course, the phenomenon of cell detachment is considered to be of paramount importance as intraperitoneal dissemination occurs by free floating cells within the ascitic fluid. In order to corroborate our hypothesis, we are currently conducting *in vitro* migration and clonogenic assays to investigate cell adhesion and motility in ovarian cancer cells either knocked down for soluble CAR isoforms by siRNAs or transfected to over-express these soluble proteins.

Furthermore, in light of the findings made by Raschperger *et al*., who showed that hCAR is predominantly expressed in epithelial cells lining the body cavities in adult mice, where it may play a role in regulating epithelial permeability and tissue homeostasis, it remains to be established whether or not hCAR and its truncated isoforms participate in ascites formation in ovarian cancer.[16] The high transcript levels of CAR variants, especially those of hCAR found by us in HPMC, also point to a role as stabilizing and regulatory constituents of TJ in the mesothelial monolayer lining the peritoneal cavity.

Interestingly, transcript levels of the soluble CAR isoforms were found to be significantly higher in ovarian carcinomas with proven p53 mutations, whereas hCAR expression was not significantly affected by p53 status. Furthermore, a positive correlation between E2F1, a proliferation-promoting transcription factor involved in the Rb pathway, and soluble CAR expression was observed. Moreover, tumors with p53 mutations have been found to be associated with high expression levels of E2F1 [Reimer *et al*., submitted for publication], and in ovarian cancer cells interferon-γ treatment resulted in down-regulation of E2F1[31] followed by a significant decline in hCAR and its soluble variants (unpublished data). These findings tempt us to speculate that E2F1 may be directly involved in the regulation of CAR. This hypothesis is further corroborated by the presence of an E2F recognition site within the CAR promoter.[37] Nonetheless, more studies are needed to understand the complex molecular background that regulates hCAR and its soluble isoforms in cancer cells.

In conclusion, data obtained in this study may point to a pivotal role of soluble CAR isoforms in the tumor biology of ovarian cancer insofar as they may counteract growth and migration inhibitory properties of the transmembrane hCAR. As shown in a training set of 140 ovarian cancer patients, especially the soluble CAR isoform 4/7 may be of major clinical relevance, possibly by promoting cancer cell dissemination. Ovarian carcinomas with high CAR 4/7 expression are consequently associated with unfavorable DFS and OS.

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