FULL PAPER

BJC

British Journal of Cancer (2013) 109, 1848–1858 | doi: 10.1038/bjc.2013.509

Keywords: Coxsackievirus and Adenovirus receptor; multitumour tissue microarray; differential expression

Presence of the Coxsackievirus and Adenovirus Receptor (CAR) in human neoplasms: a multitumour array analysis

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Background: The Coxsackie- and Adenovirus Receptor (CAR) has been assigned two crucial attributes in carcinomas: (a) involvement in the regulation of growth and dissemination and (b) binding for potentially therapeutic adenoviruses. However, data on CAR expression in cancer types are conflicting and several entities have not been analysed to date.

Methods: The expression of CAR was assessed by immunohistochemical staining of tissue microarrays (TMA) containing 3714 specimens derived from 100 malignancies and from 273 normal control tissues.

Results: The expression of CAR was detected in all normal organs, except in the brain. Expression levels, however, displayed a broad range from being barely detectable (for example, in the thymus) to high abundance expression (for example, in the liver and gastric mucosa). In malignancies, a high degree of variability was notable also, ranging from significantly elevated CAR expression (for example, in early stages of malignant transformation and several tumours of the female reproductive system) to decreased CAR expression (for example, in colon and prostate cancer types).

Conclusion: Our results provide a comprehensive insight into CAR expression in neoplasms and indicate that CAR may offer a valuable target for adenovirus-based therapy in a subset of carcinomas. Furthermore, these data suggest that CAR may contribute to carcinogenesis in an entity-dependent manner.

The Coxsackie- and Adenovirus Receptor (CAR), a transmembrane component of the tight junction complex, facilitates viral attachment onto the cellular surface, a crucial requirement for subsequent virus uptake (Bergelson *et al*, 1997; Cohen *et al*, 2001). Presence of CAR is therefore considered a critical determinant for the efficacy of therapeutic strategies employing adenoviruses. Hereby, attenuated adenoviruses, either replication-incompetent created to deliver therapeutic genes or viruses replicating restrictedly in certain cell types, may be used for the cancer treatment (Kasuya *et al*, 2007). In various human cancer types, however, particularly those displaying loss of differentiation, and advanced disease stages, reduced CAR presence has been documented (Heideman *et al*, 2001; Rauen *et al*, 2002; Sachs *et al*, 2002; Matsumoto *et al*, 2005; Korn *et al*, 2006; Anders *et al*, 2009; Wunder *et al*, 2012a, 2012b). In line with these observations, significant correlations between impaired CAR expression and a poor clinical outcome for gastric and bladder cancer patients were found (Matsumoto *et al*, 2005; Anders *et al*, 2009). Regulation of

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Received 13 May 2013; revised 5 August 2013; accepted 7 August 2013; published online 10 September 2013

declined CAR expression in cancers has been attributed to activation of the Raf/MEK/ERK pathway and the TGF-β signalling, as well as hypoxia, epithelial–mesenchymal transdifferentiation and histone deacetylation of the *CAR* gene promoter (Brüning and Runnebaum, 2003; Pong *et al*, 2003; Anders *et al*, 2003a; Lacher *et al*, 2006; Küster *et al*, 2010a,b; Lacher *et al*, 2011).

In contrast, CAR upregulation was found in cancers of the endometrium, ovary, cervix, breast and lung, as well as neuroblastomas and medulloblastomas (Martino *et al*, 2000; Martin *et al*, 2005; Persson *et al*, 2006; Wang *et al*, 2006; Reimer *et al*, 2007; Giaginis *et al*, 2008; Dietel *et al*, 2011). In breast and lung cancer types, high CAR expression has been linked to poor overall survival and shorter disease-free survival, respectively (Martin *et al*, 2005; Wunder *et al*, 2012a). Contrary to the loss of CAR in neoplasms, little is known about the molecular basis of CAR upregulation: in oesophageal squamous cell carcinoma cell lines, CAR expression was induced through the MAPK/ERK1/2 signalling, a pathway that also has been linked to CAR downregulation as described above (Ma *et al*, 2012). Furthermore, disruption of cellular organisation has been found to upregulate CAR in early breast cancer (Anders *et al*, 2003b).

Currently, it remains unclear whether these diverse results reflect entity-depending differences in CAR expression or might solely be caused by methodical differences. Nevertheless, given that differential CAR expression may indicate a progression step during malignant transformation, these previous findings might reflect the possible complex function of CAR. On one hand, loss of CAR has been suggested to decrease intercellular adhesion, promote proliferation, migration, invasion and metastatic potential of cancers, leading to the hypothesis of a tumour-suppressive role of CAR (Okegawa *et al*, 2000, 2001; Brüning and Runnebaum, 2004; Huang *et al*, 2005; Wang *et al*, 2005; Raschperger *et al*, 2006; Anders *et al*, 2009; Stecker *et al*, 2011). On the other hand, CAR has been implied to promote carcinogensesis, as increased CAR levels were found in early-stage breast cancer and breast cancer precursor cell lines (Anders *et al*, 2003b; Brüning *et al*, 2005).

Intrigued by these findings, we performed an immunohistochemical determination of CAR expression in a broad range of malignancies, corresponding precursor lesions as well as healthy controls employing tissue microarrays. Usage of this uniform methodical platform was chosen to generate data allowing for direct comparison between different organs, and hereby to identify neoplasms in which CAR expression might be of importance during malignant progression and the ones where it is not. By doing so, potential targets for adenovirus-mediated therapies based on CAR expression can be identified as well.

MATERIALS AND METHODS

Tissue microarrays and immunohistological investigations. The expression of CAR protein was assessed with immunohistochemical staining of tissue microarrays (TMA) containing a total of 3714 formalin-fixed, paraffin-embedded archival samples (diameter 0.6 mm) from a total of 100 different human tumours and preneoplastic lesions, as well as 273 corresponding controls derived from normal tissues (Simon and Sauter, 2002). All these samples (provided by RS and GS) were taken from tissues acquired for routine diagnostic purposes at the Department of Pathology, University Medical Center Hamburg-Eppendorf, in accordance with the principles of the 'Ethik-Kommission der Ärztekammer, Hamburg'. The collection and TMA-based screenings of human tumour samples were in compliance with the ethical principles for medical research issued by the World Medical Association's Declaration of Helsinki.

For the subsequent immunohistochemical study, TMA sections were deparaffinized and dehydrated, employing standard procedures using rotihistol, isopropanol and ethanol. Following common antigen-retrieval methods including trypsin and microwave treatments in 10 mM citrate buffer (pH 6.0), tissues were blocked in milk and incubated with a primary polyclonal antibody

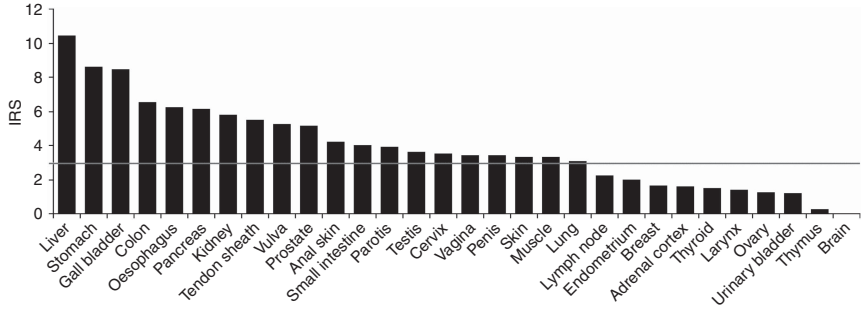

Figure 1. CAR expression in normal samples: CAR protein expression was determined with immunohistochemical staining. On the basis of the immunoreactive score, entities were considered CAR-negative (IRS 0–3) or CAR-positive (IRS 4–12) (grey line).

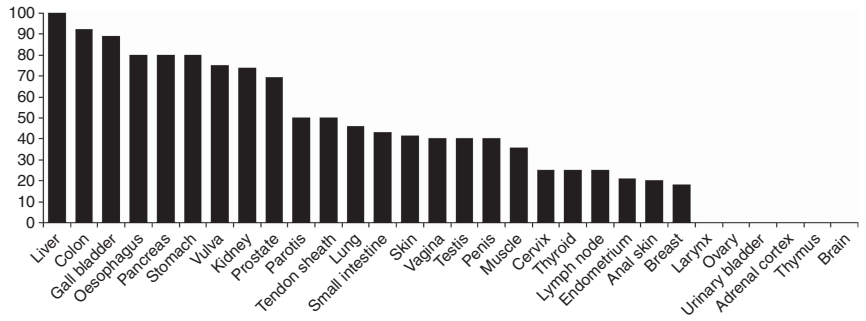

Figure 2. Percentage of CAR-positive cases in normal samples: Portion of CAR-positive cases was calculated on the basis of the IRS (see Figure 1) with 25% or less being considered low CAR-expressing entities.

Table 1. CAR immunopositivity in neoplasms and controls

Tissue type	*n*	Intensity (mean)	Pos. cells (mean)	IRS (mean)	CAR positive *n*	%	*P*-value
Respiratory tract tumours							
Larynx							
Larynx. normal	5	1.2	1.2	1.4	0	0	
Larynx. carcinoma	55	1.96	2.2	4.8	35	63.6	**0.006**
Lung							
Lung. normal	24	1.54	1.83	3.04	11	45.8	
Lung cancer. adenocarcinoma	68	2.13	2.6	5.68	56	82.4	**0.001**
Lung cancer. bronchioalveolary carcinoma	13	1.85	2.62	5.31	9	69.2	0.173
Lung cancer. large cell cancer	45	1.78	2.22	4.36	25	55.6	0.441
Lung cancer. NSCLC	10	1.3	1.9	3	4	40	0.755
Lung cancer. small cell cancer	13	1.54	1.77	3.23	6	46.2	0.985
Lung cancer. SQCC	57	2.19	2.84	6.26	48	84.2	< **0.0001**
Lymphoepithelial tumour	5	0.8	1.2	1.8	1	20	n.a.
Malignant mesothelioma	24	2.08	2.25	5.17	14	58.3	n.a.
Mucoepidermoid carcinoma	17	2.12	2.59	5.88	14	82.4	n.a.
Oral cavity. carcinoma	53	1.81	2.34	4.72	29	54.7	n.a.
Parotis							
Parotis. normal	10	1.8	1.9	3.9	5	50	
Parotis. pleomorphic adenoma	60	1.02	1.18	1.57	7	11.7	**0.003**
Warthin's tumour	54	2.76	3.13	9.2	46	85.2	**0.011**
Gastrointestinal tumours							
Colon							
Colon. normal	14	2.38	2.69	6.54	12	92.3	
Colon adenoma. low grade	45	2.49	2.27	5.82	36	80	0.301
Colon adenoma. high grade	30	2.77	2	5.63	21	70	0.112
Colon cancer	59	2.12	1.67	3.98	30	50	**0.005**
Esophagus							
Esophagus. normal	5	1.8	3.2	6.2	4	80	
Esophageal carcinoma. adenocarcinoma	59	2.29	2.24	5.19	40	67.8	0.572
Esophageal carcinoma. SQCC	56	2.41	2.48	6.09	48	85.7	0.73
Gall bladder							
Gall bladder. normal	9	2.56	3.11	8.44	8	88.9	
Gall bladder carcinoma	25	2.08	2.12	4.68	15	60	0.112
Gastrointestinal stroma tumour (GIST)	46	1.59	2.37	4.09	21	45.7	n.a.
Liver							
Liver. normal	5	2.6	4	10.4	5	100	
Hepatocellular carcinoma	53	2.43	3	7.77	45	84.9	0.349
Pancreas							
Pancreas. normal	10	2.3	2.5	6.1	8	80	
Pancreatic cancer. ductal adenocarcinoma	53	2.11	2.13	4.72	40	75.5	0.758
Pancreatic cancer. neuroendocrine	18	1.89	2.61	5.11	13	72.2	0.649
Pancreatic cancer. papilla. adeno	28	2.36	2.32	5.71	23	82.1	0.881
Small intestine							
Small intestine. normal	7	1.57	2.29	4	3	42.9	
Small intestine carcinoma	22	1.86	1.82	3.45	11	50	0.742
Stomach							
Stomach. normal	5	2.6	3.2	8.6	4	80	
Stomach cancer. diffuse type	54	1.54	1.85	3.15	20	37	0.061
Stomach cancer. intestinal type	56	1.55	2.23	3.52	22	39.3	0.078
Oncocytoma	62	2.48	2.94	7.71	54	87.1	0.654

Table 1. (Continued)

Tissue type	*n*	Intensity (mean)	Pos. cells (mean)	IRS (mean)	CAR positive *n*	%	*P*-value
Anal skin							
Anal skin. normal	5	2	2	4.2	1	20	
Anal cancer	15	1.8	2.13	3.93	7	46.7	0.292
Gynecological tumours							
Breast							
Breast. normal	11	1.18	1.18	1.64	2	18.2	
Breast cancer. apocrine carcinoma	14	1	2	2.57	3	21.4	0.84
Breast cancer. ductal carcinoma	60	0.75	0.97	1.18	5	8.3	0.314
Breast cancer. kribriform carcinoma	24	0.79	0.92	1.29	2	8.3	0.395
Breast cancer. lobulary carcinoma	64	0.42	0.5	0.59	3	4.7	0.097
Breast cancer. medullary carcinoma	63	1.17	1.62	2.32	14	22.2	0.764
Breast cancer. mucinous carcinoma	59	1.03	1.42	2.05	12	20.3	0.87
Breast cancer. phylloid carcinoma	47	0.62	0.7	0.83	2	4.3	0.101
Breast cancer. tubulary carcinoma	58	1.21	1.36	2.45	14	24.1	0.668
Cervix							
Cervix. normal	4	1.5	2	3.5	1	25	
Cervical cancer. adenocarcinoma	42	2.4	2.48	6.64	33	78.6	**0.02**
Cervical cancer. adenosquamous carcinoma	2	2	1.5	3.5	1	50	0.54
Cervical cancer. SQCC	63	2.13	2.83	6.29	52	82.5	**0.006**
Endometrium							
Endometrium. normal	19	1.26	1.32	2	4	21.1	
Endometrial cancer. endometroid carcinoma	60	2.62	2.9	7.75	55	91.7	< **0.0001**
Endometrial cancer. serous carcinoma	53	2.13	2.4	5.51	40	75.5	< **0.0001**
Ovary							
Ovar. normal	4	1.25	0.75	1.25	0	0	
Ovarian cancer. brenner tumour	40	2.05	2.38	5.53	22	55	**0.036**
Ovarian cancer. endometroid carcinoma	22	2.9	3	8.81	21	100	< **0.0001**
Ovarian cancer. mucinous carcinoma	44	2.25	3.07	7.09	44	90	< **0.0001**
Ovarian cancer. serous carcinoma	63	1.87	2.87	5.62	47	74.6	**0.002**
Teratoma	57	1.37	1.33	2.74	18	31.6	0.181
Vagina							
Vagina. normal	5	1.8	1.4	3.4	2	40	
Vagina carcinoma. SQCC	20	2.05	2.3	5	14	70	0.211
Vulva							
Vulva. normal	4	2.25	2.5	5.25	3	75	
Vulva carcinoma. SQCC	60	2.02	3.1	6.32	54	90	0.352
Genitourinary tract tumours							
Testis							
Testis. normal	5	1.8	1.8	3.6	2	40	
Testis. non-seminoma	44	1.18	1.77	2.75	13	29.5	0.631
Testis. seminoma	92	1.27	2.02	2.71	24	26.1	0.494
Penis							
Penis. normal	5	1.4	2.2	3.4	2	40	
Penile carcinoma	46	1.5	2.28	3.8	21	45.7	0.809
Prostate							
Prostate. normal	26	1.88	2.54	5.15	18	69.2	
Prostate cancer	63	0.86	1.1	1.33	5	7.9	< **0.0001**
Renal cell cancer							
Kidney. normal	19	1.95	2.68	5.79	14	73.7	
Renal cell cancer. chromophobic	56	1.95	2.98	5.95	44	78.6	0.66
Renal cell cancer. clear cell	68	1	1.38	1.62	4	5.9	<**0.0001**
Renal cell cancer. papillary	31	1.68	2.58	4.58	18	58.1	0.264
Renal cell cancer. colibri	9	0.89	2.22	2.56	3	33.3	**0.041**

Table 1. (Continued)

Tissue type	*n*	Intensity (mean)	Pos. cells (mean)	IRS (mean)	CAR positive *n*	%	*P*-value
Urinary bladder							
Urinary bladder. normal	5	1	1	1.2	0	0	
Urinary bladder cancer. non-invasive (pTa)	60	2.75	3.35	9.67	53	88.3	**<0.0001**
Urinary bladder cancer. invasive (pT2-4)	60	1.88	2.2	4.42	34	56.7	**0.015**
Urinary bladder cancer. colibri	10	1.4	2.1	2.9	3	30	0.171
Neuroendocrine tumours							
Adrenal cortex							
Adrenal cortex. normal	5	1	1.6	1.6	0	0	
Adrenal cortex. adenoma	21	1.52	2.38	3.62	8	38.1	0.097
Adrenal cortex. carcinoma	8	1.63	2.63	4.63	3	37.5	0.118
Carcinoid	38	1.92	2.26	4.47	23	60.5	n.a.
Paraganglioma	34	1.88	2.41	4.82	19	55.9	n.a.
Phaeochromocytoma	65	1.16	1.73	2.5	16	25	0.202
Thyroid							
Thyroid. normal	4	1	0.75	1.5	1	25	
Thyroid carcinoma. anaplastic	3	1.67	1	1.67	0	0	0.35
Thyroid carcinoma. follicular	46	2.5	2.63	7.13	37	80.4	**0.013**
Thyroid carcinoma. medullary	25	2.24	2.08	4.6	15	60	0.191
Thyroid carcinoma. papillary	47	2.51	2.62	7.13	40	85.1	**0.004**
Thyroid. adenoma	62	2.82	2.89	8.3	56	90.3	< **0.0001**
Hematological neoplasias							
Lymph node							
Lymph node. normal	20	1.1	1.25	2.2	5	25	
Hodgkin's lymphoma	38	1.29	1.5	1.95	5	13.2	0.256
Non-Hodgkin's lymphoma	8	1.38	1.25	2.13	1	12.5	0.466
Thymus							
Thymus. normal	4	0.25	0.25	0.25	0	0	
Thymoma	55	1.24	1.76	2.84	20	36.4	0.138
Neuronal tumours							
Brain							
Brain. normal	5	0	0	0	0	0	
Astrocytoma	37	1.11	1.68	2.46	11	29.7	0.156
Ependymoma	10	1.1	1.1	1.4	1	10	0.464
Medulloblastoma	4	1.75	1.5	2.75	2	50	0.073
Oligodendroglioma	23	1.39	1.52	2.74	7	30.4	0.154
Neuroblastoma	48	1.88	1.9	4.17	26	54.2	**0.021**
Soft tissue tumours							
Muscle							
Muscle. normal	14	1.64	1.79	3.29	5	35.7	
Angiosarcoma	7	1.57	1.43	2.43	2	28.6	0.743
Dermatofibrosarcoma protuberans	5	0.8	1	1.4	1	20	0.516
Carcinosarcoma	36	0.94	1.58	1.92	7	19.4	0.226
Desmoid tumour	9	0.44	0.67	0.67	0	0	0.043
Tendon sheat							
Tendon sheat. normal	2	2	2.5	5.5	1	50	
Giant cell tumour of the tendon sheat	33	2.03	2.3	4.73	24	72.7	0.49
Granular cell cancer	7	1.29	1.71	2.43	2	28.6	n.a.
Haemangiopericytoma	7	1.29	1.57	2.43	2	28.6	n.a.
Leiomyoma	24	1.29	1.96	3.17	11	45.8	0.542
Leiomyosarcoma	28	1.36	2.25	3.61	12	42.9	0.657
Liposarcoma	16	0.81	1.38	1.5	2	12.5	n.a.
Malignant fibrous histiocytoma	24	1.08	1.42	2.21	4	16.7	n.a.
Malignant schwannoma	14	1.14	1.14	2	2	14.3	n.a.
Neurofibroma	49	1.02	1.12	2.04	10	20.4	n.a.
Stroma sarcoma	11	1	1.64	2.09	3	27.3	0.653

Table 1. (Continued)

Tissue type	*n*	Intensity (mean)	Pos. cells (mean)	IRS (mean)	CAR positive *n*	%	*P*-value
Bone tumours							
Chondrosarcoma	4	1.75	2	4.25	2	50	n.a.
Skin tumours							
Skin							
Skin. normal	17	1.65	1.76	3.29	7	41.2	
Basal cell adenoma	34	1.56	1.59	2.74	14	41.2	1
Basalioma	46	2.54	3.04	7.91	42	91.3	**< 0.0001**
Malignant melanoma	30	1.43	1.83	2.9	9	30	0.437
Merkel cell cancer	2	1	1	1	0	0	0.253
Naevus							
Naevus. benign	40	2.08	2.08	4.55	24	60	0.192
Pilomatrixoma	38	1.39	1.55	2.82	16	41.2	0.949
Skin cancer. SQCC	45	1.6	2.36	3.82	22	48.9	0.587

NOTE. All tissue samples were derived from surgically removed specimens. Results were calculated either by Fisher's exact test or by Chi-square test when applicable. Significant results for differential presence compared with available normal controls are shown in bold.

against CAR (1:50, H-300: sc-15405, Biotechnology Inc., Santa Cruz, CA, USA) for 16 h at 4 °C. Subsequently, sections were incubated in biotinylated goat anti-rabbit immunoglobulin (1:400; Vector Laboratories, Burlingame, CA, USA), followed by treatment with the streptavidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). Using diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Munich, Germany), sections were developed in hydrogen peroxide/PBS and counterstained with haemalaun. Immunostainings of CAR were analysed by two pathologists (DG and MV) blinded to clinicopathological data and scored according to (a) percentage of CAR-immunopositive cells ('0': 0%, '1': <10%, '2': 11–50%, '3': 51–80 %, '4': 81–100%) and (b) staining intensity ('0': no specific signal, '1': weak, '2': medium, '3' strong). On the basis of these data, the immunoreactive score (IRS) was calculated by percentage of positive cells × staining intensity score. For further evaluation, an IRS from 0 to 3 was considered CAR-negative, whereas 4–12 was regarded as CAR-positive.

Statistical methods. Statistical calculations using (Fisher's exact probability test or χ^2 test, respectively) were performed using the SPSS software (version 11.5; SPSS Inc, Chicago, IL, USA).

RESULTS

Expression of CAR in carcinomas and corresponding normal tissues. Immunopositivity of CAR was found in normal samples of all entities, except of the brain. Expression levels, however, displayed a high variability ranging from abundant presence in the liver, stomach and gall bladder to barely detectable, such as in the thymus (Figure 1). Highest percentages of CAR-positive tissues (IRS >3) were seen in the liver, colon, gall bladder, oesophagus, pancreas, stomach and vulva. On the other hand, low counts of CAR-positive cases (maximum of 25%) were noted in the cervix, thyroid, lymph nodes, endometrium, anal skin and breast. No cases with an IRS >3 were observed in the larynx, ovary, urinary bladder, adrenal cortex, thymus and brain (Figure 2).

In neoplasias, a great degree of diversity of CAR expression was notable as well, with ubiquitous CAR expression in several early stages of malignant transformation such as non-invasive urinary bladder cancer, Warthin Tumours, thyroid adenoma and basalioma. In advanced stages, high CAR expression levels were detected, for instance, in hepatocellular and endometroid carcinomas. In contrast, low CAR expression levels were found in prostate cancer, various subtypes of breast cancer, Merkel cell carcinoma and desmoid tumours (Table 1).

In comparison with healthy controls, significantly increased numbers of CAR-positive cases were found in basalioma, larynx carcinoma, Warthin's tumour, lung cancer, cervical cancer, endometrial cancer, ovarian cancer, urinary bladder cancer, thyroid adenoma and carcinoma, as well as in neuroblastoma (Table 1; Figure 3). On the other hand, significantly lower CAR expression levels were seen in pleomorphic adenoma of the parotid gland, colon cancers, prostate cancers, as well as subtypes of renal cell cancers (Table 1; Figure 4). To assess whether CAR presence correlates with clinicopathological parameters, we compared our findings for CAR immunopositivity with tumour grade (G), local tumour growth (T-category) and nodal status (N-category) where applicable, revealing the loss of CAR in locally advanced colon cancers (Table 2).

DISCUSSION

Our data provide insight into CAR expression levels in a broad range of neoplasias and their corresponding normal tissues, including several that have not been investigated before. As the samples in our analysis were all stained in one procedure, the results allow for a direct comparison between different entities for the first time. It reveals considerable differences in CAR expression levels and confirms the hypothesis of entity-specific expression pattern. Hereby, our data may provide a basis to gain further insight into the complex and potentially organ-site-specific function and regulation of CAR during carcinogenesis. Furthermore, entities with high CAR presence identified by our study may pose promising targets for therapeutic adenoviruses.

In normal tissues, our observations of high CAR expression level within the liver, stomach, colon and pancreas are in agreement with previous reports (Korn *et al*, 2006; Anders *et al*, 2009; Stecker *et al*, 2011). Our finding of profuse immunopositivity within the

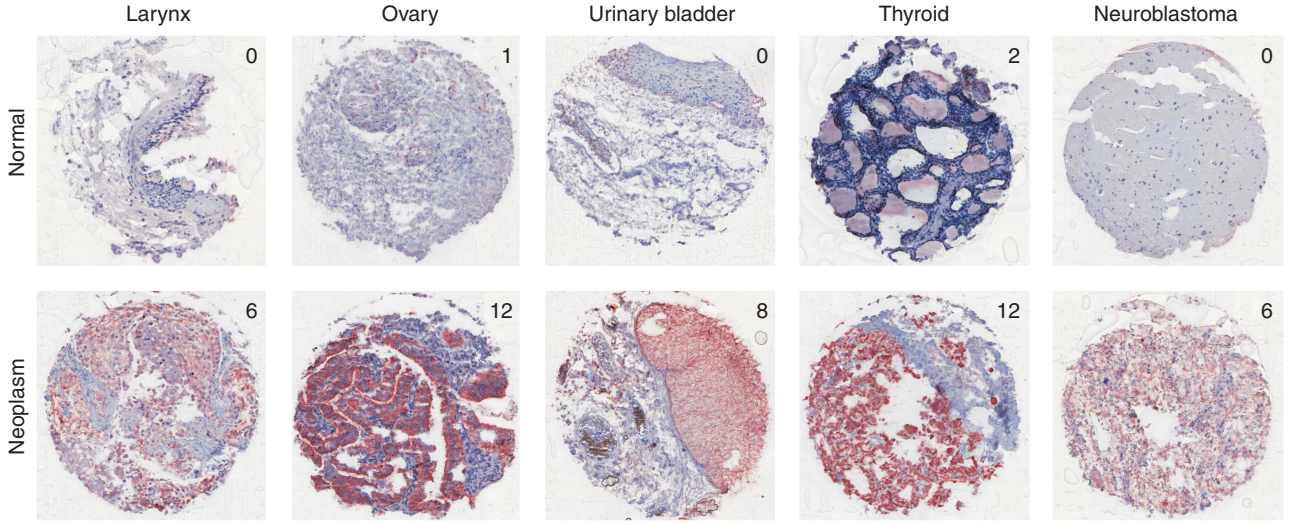

Figure 3. CAR overexpression in neoplasms: Representative examples of entities displaying elevated CAR protein expression compared with respective normal controls (numbers = IRS of the individual specimen). Ovary: endometroid carcinoma; urinary bladder cancer: non-invasive/pTa; thyroid: papillary carcinoma.

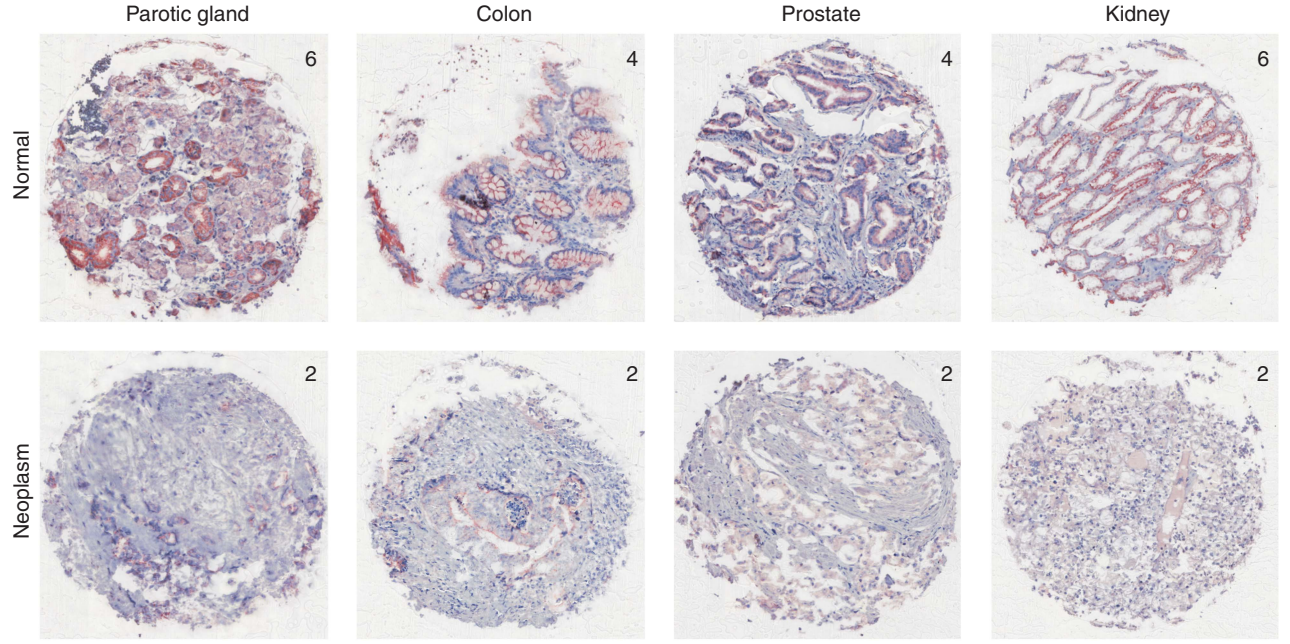

Figure 4. Loss of CAR expression in neoplasms: Typical sites with significant down regulation of CAR protein expression (numbers = IRS of the individual specimen). Kidney: clear cell renal cancer.

gall bladder marks the first description of this phenomenon to our best knowledge. These data suggest a particular impact of CAR within the gastrointestinal tract. In line with this hypothesis, functional CAR knockout in a murine model led to a dilated intestinal tract (Pazirandeh *et al*, 2011). Despite abundant CAR presence, symptomatic infections of these organs by Adeno- and Coxsackieviruses are rare because of the limited access to CAR and acquired immunity. Nevertheless, high CAR expression within the liver may lead to substantial unwanted sequestering of systemically administered therapeutic adenoviruses (Arnberg, 2012). In contrast, we found no detectable CAR immunopositivity within the brain, in line with previous studies showing the white matter being CAR-negative and scattered CAR-positive neurons within the neocortex and in ependymal cells only (Johansson *et al*, 1999; Persson *et al*, 2006).

In several early neoplasms, we did note significantly elevated CAR expression levels. Hereby, our finding of increased CAR expression in basaliomas, thyroid adenomas and Warthin's tumours – benign neoplasms of the salivary glands – are the first description of this fact to our best knowledge. The later might be of particular interest, as we did note a significant impairment of CAR in pleomorphic adenoma of the parotid gland also. The functional impact of this result, however, remains to be elucidated.

In cancer types, our finding of significant CAR increase in laryngeal carcinoma marks the first description of this phenomenon to our best knowledge. Therefore, our data may initiate further studies in this entity. In line with prior reports, we noted abundant CAR presence in several subtypes of thyroid carcinoma (Marsee *et al*, 2005; Giaginis *et al*, 2010), in lung cancer (Wang *et al*, 2006; Chen *et al*, 2013), as well as in neuro- and

Table 2. CAR presence and clinico-pathological parameters

	pT					pN					G				
Tissue type	**1**	**2**	**3**	**4**	***P*-value**	**0**	**1**	**2**	**3**	***P*-value**	**1**	**2**	**3**	**4**	***P*-value**
Larynx. carcinoma					0.283					0.749					0.302
CAR–	0	2	2	4		5	1	0	0		1	8	1		
CAR+		3	8	6	4		9	2	2	1		0	20	3	
Lung cancer. adenocarcinoma					0.581					0.073					0.452
CAR–	2	5	0	0		0	5	1	0			3	4		
CAR+	9	13	3	4		11	6	6	1			13	9		
Lung cancer. bronchioalveolary carcinoma					0.956					0.307					0.018
CAR–	1	2				3	0	0			2	0	0		
CAR+	2	4				2	2	1			0	4	2		
Lung cancer. large cell cancer					0.274					0.222					0.147
CAR–	0	2	1	0		1	1	1					3	0	
CAR+	1	6	2	2		4	4	3					2	2	
Lung cancer. NSCLC					0.664					0.444					0.816
CAR -	3	3	1	1		5	1	0	0			6	2		
CAR +	7	23	6	3		15	14	6	1			26	7		
Colon cancer					**0.010**										
CAR–	0	3	14	12											
CAR+	2	8	8	5											
Cervical cancer. adenocarcinoma					0.513					0.457					0.112
CAR–	5	0				3	0				2	0	1		
CAR+	18	4				10	5				2	7	5		
Cervical cancer. SQCC					0.248					0.966					0.662
CAR–	10	1		0		7	2					4	7		
CAR+	31	12		3		31	11					13	31		
Endometrial cancer. endometroid					0.646					0.535					0.922
CAR–	5	0	0			1	1				3	1	1		
CAR+	41	7	5			20	4				29	15	9		
Ovarian cancer. mucinous					0.438					0.803					0.275
CAR–	0	0				0	0				0	2	0		
CAR+	11	1				3	1				9	9	4		
Ovarian cancer. serous					0.312					0.876					0.120
CAR–	1	0	3			3	5				1	2	12		
CAR+	1	3	17			11	12				2	20	25		
Renal cell cancer. chromophobic					0.523										0.553
CAR–	3	2	1								0	5	1	0	
CAR+	16	10	6								5	23	2	2	
Renal cell cancer. colibri					0.337					0.392					0.809
CAR–	2	1	3			2	1	1				3	1		
CAR +	0	1	1			0	2	0				2	1		
Renal cell cancer. clear cell					0.949					0.465					0.635
CAR–	46	3	13			16	2				8	45	8		
CAR+	3	0	1			0	0				0	3	1		
Renal cell cancer. papillary					0.123					0.100					0.495
CAR–	9	2	2			1	2				3	9	1		
CAR+	12	2	0			0	0				5	11	0		
Urinary bladder cancer. colibri					0.724					0.665					
CAR–	1	1	2	2		5	1	1							
CAR+	0	1	1	0		2	0	1							
Urinary bladder cancer					0.622					0.101					0.234
CAR–		21	5	0		1	2	0				3	23		
CAR+		28	5	1		0	0	3				8	26		
Thyroid carcinoma. follicular					0.468										
CAR–	7	2	0	0											
CAR +	29	3	4	1											

Table 2. (Continued)

	pT					pN					G				
Tissue type	**1**	**2**	**3**	**4**	***P*-value**	**0**	**1**	**2**	**3**	***P*-value**	**1**	**2**	**3**	**4**	***P*-value**
Thyroid carcinoma. medullary					0.551					0.672					
CAR−	0	2	1	0		0	1								
CAR+	1	4	0	1		1	2								
Thyroid carcinoma. papillary					0.383					0.673					0.386
CAR−	0	4	1	0		0	1				1	0			
CAR+	8	16	4	7		4	6				1	1			

NOTE. Results were calculated by Chi-square test for neoplasms showing differential CAR presence compared with respective controls. Significant results are shown in bold.

Table 3. Findings of differential CAR presence in human neoplasms compared with previous publications

CAR upregulation		
Basalioma	N	—
Thyroid adenoma	N	—
Warthin's tumours	N	—
Laryngeal cancer	N	—
Thyroid carcinoma	A	(Marsee *et al*, 2005; Giaginis *et al*, 2010)
Lung cancer	A	(Wang *et al*, 2006; Chen *et al* 2013)
Neuroblastomas	A	(Persson *et al*, 2006)
Medulloblastomas	A	(Persson *et al*, 2006)
Endometrial cancer	A	(Giaginis *et al*, 2008)
Ovarian cancer	A	(Reimer *et al*, 2007)
Cervical carcinoma	A	(Dietel *et al*, 2011)
Non-invasive urinary bladder cancer	D	(Sachs *et al*, 2002; Matsumoto *et al*, 2005; Buscarini *et al*, 2007)
CAR downregulation		
Pleomorphic adenoma (parotid gland)	N	—
Colon	A	(Korn *et al*, 2006; Zhang *et al*, 2008; Stecker *et al*, 2011)
Prostate	A	(Rauen *et al*, 2002)
Kidney	A	(Okegawa *et al*, 2001)

Abbreviations: A = agreement; D = disagreement; N = new finding.

medulloblastomas (Persson *et al*, 2006). Moreover, in agreement with previous reports we found significantly hightened CAR presence in cancers of the endometrium (Giaginis *et al*, 2008), ovary (Reimer *et al*, 2007) and cervix (Dietel *et al*, 2011). These data suggest that CAR overexpression occurs preferentially in cancers of the female reproductive system, contrary to reduced CAR presence in neoplasms of the testis and prostate. The reason for this phenomenon remains unclear, yet hormone-driven effects might be of particular interest. Previously, an increased CAR expression by treatment with estradiol was found in hormone receptor-positive breast cancer and ovarian cancer cell lines (You *et al*, 2001; Auer *et al*, 2009). Furthermore, our data imply that CAR may have little impact on breast cancer as we did not observe distinct expression changes in breast epithelium in contrast to a previous study, describing elevated transcriptional CAR expression in breast cancers (Martin *et al*, 2005).

In disagreement with previous reports we noted a significantly increased CAR presence in non-invasive urinary bladder cancers because of the low presence of CAR in normal urinary bladder samples (Sachs *et al*, 2002; Matsumoto *et al*, 2005; Buscarini *et al*, 2007). These differences might be caused by methodical differences such as the use of different antibodies, yet may be explained by the limited number of healthy cases in our study also.

Concerning CAR downregulation, our finding in cancer types is in agreement with previous studies for the colon (Korn *et al*, 2006; Zhang *et al*, 2008; Stecker *et al*, 2011), prostate (Rauen *et al*, 2002) and kidney (Okegawa *et al*, 2001).

For a subset of entities, access to clinicopathological data allowed for further analysis of potential relations to CAR presence. Our finding of CAR downregulation in locally advanced colon cancers underlines the concept of CAR's tumour-suppressive role in this entity (Stecker *et al*, 2011). However, as our study aims for a comprehensive evaluation of neoplasms, it is limited although concerning sample numbers and clinicopathological data for individual entities. Therefore, our study potentially underestimates associations between CAR and clinicopathological properties.

In conclusion, our data suggest that differential expression of CAR in cancer types represents an entity-specific phenomenon with CAR upregulation happening more frequently than its downregulation. These findings shed a new light on CAR regulation in cancer types also. To date, mainly CAR downregulation in cancer types has been investigated. Hereby, activation of the Raf/MEK/ERK pathway and TGF-β signalling, hypoxia, epithelial–mesenchymal transdifferentiation and histone deacetylation of the *CAR* gene promoter were identified as regulators of CAR expression (Brüning and Runnebaum, 2003; Pong *et al*, 2003; Anders *et al*, 2003a; Lacher *et al*, 2006; Küster *et al*, 2010a, 2010b; Lacher *et al*, 2011). In contrast, few studies have investigated the mechanism of CAR upregulation. Therefore, it remains to be elucidated whether the MAPK/ERK1/2 signalling induces CAR expression in other entities than oesophageal squamous cell carcinomas (Ma *et al*, 2012), and, for instance, whether hormones influence CAR levels in cancer types as discussed above. Furthermore, our findings have potential implications for the understanding of the function of CAR in cancer types. To date, CAR has been mainly attributed cancer-suppressive properties. Previous studies on CAR function, however, have been performed in models of advanced cancer types of the colon, prostate and kidney. However, all these entities do belong to the limited number of sites showing significant CAR downregulation in our study. Upregulation of CAR on the other hand might be suggestive of a tumour-promoting function of CAR in several other organs. In line with this hypothesis, an association has been found between high CAR expression and increased proliferation and/or invasion in endometrial, ovarian, and cervical cancers as well as in lung cancer (Brüning *et al*, 2005; Giaginis *et al*, 2008; Dietel *et al*, 2011; Chen *et al*, 2013). Furthermore, CAR has been shown to foster early carcinogenesis in ovarian and cervical cancers, with CAR-expressing cell lines displaying less sensitivity towards apoptotic stimuli (Brüning *et al*, 2005). On the other hand, migration and

metastatic phenotypes are being suppressed by CAR overexpression in cell lines derived from the same entities (Brüning and Runnebaum, 2004; Wang *et al*, 2005). These results underline that off course CAR expression levels *per se* do not allow for prediction of functional impact. Nevertheless, our findings may serve as a guide to neoplasms potentially influenced by CAR.

ACKNOWLEDGEMENTS

We thank Jennifer Schröder-Schwarz for technical assistance. This study was supported by a grant from the Deutsche Forschungsgemeinschaft to MA.

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