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Expression of coxsackie and adenovirus receptor is correlated with inferior prognosis in liver cancer patients

XUE YANG[1,2*], SHUANGSHUANG LI[1,2*], HUIJU WANG[1,3], WANYUAN CHEN[4], XIAOZHOU MOU[1,2] and SHIBING WANG[1,2]

[1]Clinical Research Institute, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College; [2]Key Laboratory of Tumor Molecular Diagnosis and Individualized Medicine of Zhejiang Province; [3]Key Laboratory of Gastroenterology of Zhejiang, Zhejiang Provincial People's Hospital; [4]Department of Pathology, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China

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Abstract. The coxsackie and adenovirus receptor (CAR), a tumor suppressor, is vital for the effectiveness of therapies which utilize the adenovirus. However, studies on CAR expression in hepatocellular carcinoma (HCC) are conflicting and its clinical significance requires exploration. In this study, immunohistochemistry has been carried out on tissue microarrays consisting of 198 pairs of HCC and neighboring healthy tissue specimens from Chinese Han patients to evaluate CAR expression. Relative to normal tissues, decreased CAR expression (56% vs. 57%; P>0.05) was detected in HCC samples. CAR immunopositivity in tumors was not dependent upon sex, age, tumor dimensions, differentiation, TNM stage or metastasis in HCC patients; however, positive expression was observed in 56% of the samples from patients with hepatic metastasis, which was the same as those devoid of metastasis (56%; P=0.042). Furthermore, survival analysis confirmed that the expression of CAR revealed no correlation with the prognosis. It was established that CAR exerted complex effects during liver tumorigenesis, potentially based on the stage of the cancer. Therefore, CAR expression analysis has to be carried out prior to adenoviral oncolytic therapy to stratify the patients.

Correspondence to: Dr Shibing Wang, Clinical Research Institute, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, 158 Shangtang Road, Hangzhou, Zhejiang 310014, P.R. China
E-mail: wangshibing@hmc.edu.cn

*Contributed equally

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Introduction

Hepatocellular carcinoma (HCC) is a common solid tumor displaying inferior prognosis and a high recurrence rate, which results in extensive worldwide mortality (1). Due to the development of innovative treatments like hepatic transplantation, hepatic resection, chemotherapy, and radiofrequency ablation, the survival rates of patients have improved (2); however, an effective treatment for the complete cure of HCC has yet to be developed. Although significant efforts have been made to elucidate the disease progression and to develop valuable therapies, the molecular mechanisms in HCC are still mostly unknown. Hence, there is an imperative need to enhance the prognosis of the disease. Oncolytic viruses (OVs), which can be engineered to selectively replicate intracellularly and destroy tumor tissues, have been applied as an efficacious solution against tumors. Numerous OVs have been designed to exploit their antitumor effects; for instance, pexastimogene devacirepvec (Pexa-Vec) is currently in a phase III trial for the treatment of HCC (3). In this context, the safety and efficacy of adenovirus vectors for gene delivery should also be demonstrated in the future.

The coxsackie and adenovirus receptor (CAR), a transmembrane constituent of the tight junctions in epithelial tissue, has been originally discovered as a viral attachment site that is essential for virus uptake (4). Walters *et al* (5) established the role of CAR in gene transfer and as a principal receptor for the coxsackie B virus and adenovirus. Pandha *et al* (6) demonstrated that CAR expression was strongly correlated with adenovirus infection, adhesion and transgene expression. Attenuated adenoviruses, which may be replication-incompetent to transmitted therapeutic genes or viruses, can be employed in cancer therapy (7). Therefore, the expression of CAR is considered a crucial factor for the effectiveness of adenovirus-based therapeutics.

Analysis of CAR expression in several classes of tumors produced diverse results. It has been reported that CAR expression was low in colon, lung and bladder (8-11) tumors, predominantly in poorly differentiated and advanced-stage cancers (9,12,13). Additionally, downregulated CAR expression predicted an inferior clinical result for gastric and bladder cancer patients (9,14). Contrastingly, CAR upregulation has been detected in endometrial, ovarian, cervical and breast cancers, along with neuroblastomas and medulloblastomas (15-20). Moreover, elevated CAR expression was correlated with inferior prognosis in breast and lung cancers (12,21). Therefore, there is a need to confirm if these outcomes indicate a disparity in CAR expression or a result from racial and procedural variations.

In the present investigation, immunohistochemistry was employed to evaluate CAR expression in HCC and neighboring healthy tissue specimens in tissue microarrays (TMAs). A greater sample size was chosen to acquire data for an improved understanding of the function of CAR in the progression of HCC. Additionally, probable targets for adenovirus-mediated therapeutic strategies related to CAR expression were established.

Materials and methods

HCC patients in tissue microarray. HCC protein expression levels were assessed with immunohistochemical staining of tissue microarrays, which were purchased from Shanghai Biochip Co., Ltd. (Shanghai, China). The patient samples were collected from Zhejiang Provincial People's Hospital (Hangzhou, China) and all patients provided written informed consent. The study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital. The TMAs contained a total of 396 formalin-fixed, paraffin-embedded archived samples from a total of 198 HCC patients from the Chinese Han population, in addition to 198 corresponding controls derived from adjacent normal tissue samples.

The patient cohort consisted of 159 males and 39 females, with a median age of 55 years (range, 27-91 years) at the time of surgery. All patients had follow-up records for >5 years. The survival time was calculated from the date of surgery to the follow-up deadline or mortality.

Immunohistochemical staining. Immunohistochemical staining was performed according to the standard method. Briefly, 5-μm sections from the TMAs were baked at 70˚C for 2 h. Then, the sections were de-paraffinized in xylene, rehydrated using a gradient of ethanol concentrations, boiled in 1 mM TE buffer with a high-pressure cooker for 3 min for antigen retrieval, blocked with 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity and incubated with 10% goat non-immune serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 20 min to reduce background non-specific staining. Subsequently, TMA sections were incubated with rabbit anti-human primary polyclonal antibody against CAR (dilution, 1:400; cat. no. Sc-15405; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C, and then incubated with biotin-labeled secondary antibody (1:200; cat. no. AB-2548649) at room temperature for 15 min, followed by incubation with HRP-conjugated streptavidin (1:100; cat. no. AB-11155398; both Invitrogen; Thermo Fisher Scientific, Inc) at room temperature for 15 min. Then, color development was performed with a DAB Substrate kit (Dako, Glostrup, Denmark). Finally, the sections were counterstained with hematoxylin, dehydrated, cleared and mounted.

Evaluation of the immunohistochemical stainings. Immunohistochemical stainings of CAR were scored by two pathologists independently, based on the intensity and the proportion of positively stained cells. Staining intensity was evaluated with a four-tiered grading system: 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells were scored as follows: 0, no cells stained, 1, 1-25% of cells stained; 2, 26-50% of cells stained; 3, 51-75% of cells stained; and 4, >75% of cells stained. Scores for intensity and percentage of positive cells were multiplied. Scores ≤6 were used to define tumors with low CAR expression and scores >6 with high CAR expression.

Statistical analysis. Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) software 13.0 (SPSS Inc., Chicago, IL, USA). The χ^2-tests were applied to assess the statistical significance of the associations between CAR expression and clinicopathological parameters, respectively. Cox proportional hazards regression model was used to perform multivariate survival analysis to assess predictors associated with prognosis. P<0.05 was considered to indicate a statistically significant difference.

Results

CAR expression in HCC is inferior to that in adjacent healthy tissue samples. Immunohistochemistry was employed to evaluate the distribution of HCC, where immunostaining of the tissues predominantly affected the cell membrane and cytoplasm (Fig. 1). Positive CAR expression was observed in 114 out of 198 (57%) normal liver tissue samples, which was slightly superior to that of HCC (56%, 111/198, χ^2=174.7, P>0.05).

Patient characteristics and the association between CAR expression and clinicopathological parameters of HCC. With the aim of exploring whether CAR is related to HCC progression, the expression of CAR was studied with respect to clinicopathological features in HCC. The outcomes established that the occurrence of CAR immunopositivity was not significantly dependent upon sex, age, metastasis, microvascular invasion, HBs antigen, cirrhosis and AFP (Table I).

Survival analysis reveals that CAR immunopositivity does not significantly diminish survival times. The average survival time of CAR-positive liver cancer patients (42.201±4.056 months) was not significantly different than that of CAR-negative patients (40.934±3.409 months, P=0.240). The Kaplan-Meier survival curve indicated that CAR expression was not significantly associated to overall survival in HCC patients (Fig. 2). Additionally, the prognosis factors of HCC were analyzed by Cox-regression analysis. Univariate analysis indicated that tumor size (P=0.005), metastasis (P<0.001), microvascular invasion (P=0.016), Edmondson grade (P<0.001) and AFP (P=0.002) were independent prognostic factors in patients with HCC. Multivariate analysis revealed that distant metastases (P<0.001), tumor number (P=0.001), Edmondson grade (P=0.028) and AFP (P=0.008) were independent prognostic factors for patients with HCC (Table II).

Table I. Expression of CAR in HCC tissues.

		CAR expression		
Clinical parameters	No.	Low	High	P-value
Age, years				0.989
<55	75	33	42	
≥55	123	54	69	
Sex				0.303
Male	159	67	92	
Female	39	20	19	
Size, cm				**0.019**
<5	100	36	64	
≥5	95	50	45	
Tumor number				0.407
Single	161	73	88	
Multiple	37	14	23	
Edmondson grade				**0.009**
I+II	127	47	80	
III	71	40	31	
Metastasis				0.987
M0	182	80	102	
M1	16	7	9	
Microvascular invasion				0.880
Absence	65	33	32	
Presence	73	38	35	
HBs antigen				0.744
Negative	43	18	25	
Positive	150	67	83	
Cirrhosis				0.701
Negative	62	26	36	
Positive	136	61	75	
AFP				0.274
<50	101	44	57	
≥50	97	49	48	

Bold indicates a statistically significant result. CAR, coxsackie and adenovirus receptor; HCC, hepatocellular carcinoma.

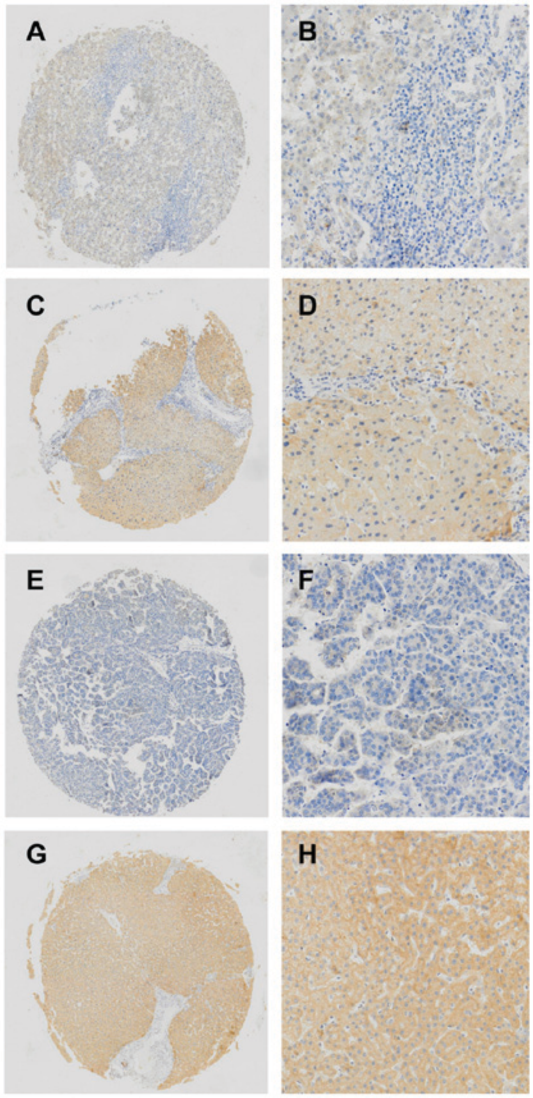

Figure 1. Immunohistochemical analysis of CAR in liver cancer and normal tissues. (A and B) Low expression of CAR in normal liver tissues adjacent to cancerous tissues. (C and D) High expression of CAR in normal liver tissues adjacent to cancerous tissues and positive staining, primarily in the membrane and cytoplasm. (E and F) Low expression of CAR in the tumor sample. (G and H) High expression of CAR in the tumor sample and positive staining, mainly in the membrane and cytoplasm. (A, C, E, and G) Original magnification, x200; (B, D, F, and H) original magnification, x800. CAR, Coxsackie and adenovirus receptor.

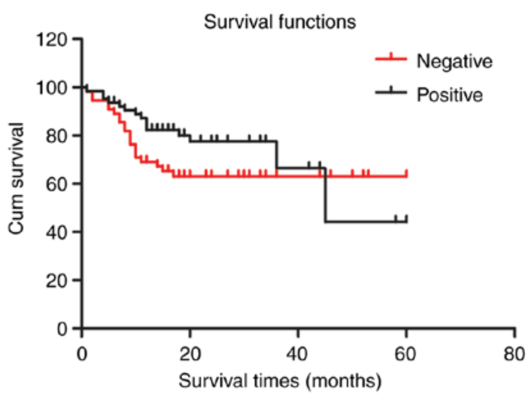

Figure 2. Estimation of Kaplan-Meier survival curves in CAR-positive and CAR-negative patients indicating that CAR expression displayed no notable association with overall survival.

Discussion

HCC is a common lethal cancer which leads to large-scale mortality worldwide (22). At present, there are numerous limitations to the treatment of HCC; hence, there is a necessity for the development of innovative and efficient therapeutics (23-25). It has been reported that low expression of CAR was detected in colon, lung and bladder (8-11) tumors and downregulated CAR expression predicted an inferior clinical result for gastric and bladder cancer patients (9,14). Conversely, CAR upregulation has been detected in endometrial, ovarian, cervical, breast cancers, along with neuroblastomas and medulloblastomas (15-20). Moreover, increased CAR expression was correlated with inferior prognosis in breast and

Table II. Univariate and multivariate Cox-regression analyses of the clinicopathological features in HCC patients.

		Univariate analysis			Multivariate analysis		
Parameters	No.	HR	95.0% CI for HR	P-value	HR	95.0% CI for HR	P-value
Age (<55 years/≥55 years)	75/123	0.651	0.409-1.038	0.071	0.335	0.119-0.943	0.058
Sex (male/female)	159/39	1.575	0.922-1.575	0.096	0.623	0.202-1.922	0.410
Tumor size (<50 mm/≥50 mm)	100/95	1.954	1.219-3.132	**0.005**	1.425	0.423-4.799	0.567
Tumor number (single/multiple)	161/37	1.239	0.678-2.264	0.487	11.26	2.573-49.279	**0.001**
Metastasis(M0/M1)	182/16	4.821	2.551-9.112	**<0.001**	26.004	5.762-7.352	**<0.001**
Microvascular invasion (-/+)	65/73	1.89	1.127-3.171	**0.016**	0.688	0.216-2.194	0.527
Edmondson grade (I+II/III)	127/71	2.783	1.735-4.464	**<0.001**	2.984	1.124-7.923	**0.028**
Cirrhosis (-/+)	62/136	1.153	0.698-1.905	0.577	3.52	0.949-10.052	0.06
AFP (<50 μg/l/≥50 μg/l)	101/97	2.51	1.395-4.517	**0.002**	4.245	1.455-12.386	**0.008**
CAR expressions (-/+)	88/110	0.667	0.345-1.289	0.228	0.677	0.206-2.225	0.520

Bold indicates a statistically significant result. HCC, hepatocellular carcinoma; HR, hazard ratio; CAR, coxsackie and adenovirus receptor; CI, confidence interval.

lung cancers (12,21). However, in the present study, relative to normal tissues, slightly decreased CAR expression was detected in HCC samples and the Kaplan-Meier survival curve indicated that CAR expression was not significantly associated to overall survival in HCC patients. CAR immunopositivity in tumors was not dependent upon sex, age, differentiation or metastasis in HCC patients, which was in agreement with earlier observations by Stecker *et al* (26). Also, positive expression was observed in 56% of the samples from patients with hepatic metastasis, which was the same as those devoid of metastasis. Although, the relevant molecular mechanism is important, a limitation of this study is that it lacks some functional experiments to study the relevant mechanisms. In subsequent studies, with the purpose of exploring the relevant molecular mechanisms of CAR, CAR expression will be altered (downregulation and upregulation), followed by the detection of the expression of related genes through fluorogenic quantitative PCR and western blotting.

Recently, cancer virotherapy, principally mediated by oncolytic viruses (OVs), has gained popularity as a new and potent strategy in the field of cancer therapy (27-29). Consequently, the safety and efficacy of adenovirus vectors for gene delivery should also be illustrated in the future. The first OV therapy for cancer was approved in 2015 after more than a century of extensive research. However, this is considered as a modest victory when compared to the numerous small molecule anticancer agents and antibody therapies that have gained approval for clinical use over the previous thirty years. Although OVs are markedly effective as anticancer agents with low toxicity *in vitro* and *in vivo*, their efficacy as a single agent therapy is limited. In this context, a combination of gene therapy and therapeutically valuable OVs, such as oncolytic adenoviruses (OAds), is one of the most potent therapeutic approaches. Furthermore, OAds have been genetically improved to take advantage of the altered tumor environment.

In the last twenty years, viral and non-viral vector-mediated gene therapy has been established as a potential therapeutic strategy for a range of cancers and other serious diseases which would otherwise be deemed incurable with traditional drugs (28). Previously, several trials focusing on the combination of OAds with immunostimulatory-, proapoptotic- or tumor suppressor genes were carried out and revealed enhanced antitumor potency (28,30-33). Clinically detected cancers may have escaped antitumor immune mechanisms during their growth; hence, the probability of potent immunotherapies for their cure is currently becoming a clinical reality (34). Furthermore, a circadian crosstalk exists among OAds, TRAIL and IL-12 in the stimulation of antitumor immunity (35). Angiogenesis is vital for tumorigenesis; accordingly, several studies have ascertained that OAds can impair tumor-mediated angiogenesis (36). Apoptosis is a programmed cell death process which eliminates cancer cells and other detrimental cells to sustain homoeostasis; thus, it presents a potential target for innovative cancer therapeutics. In this context, a combination therapy of OAd-expressing TRAIL with an additional type of immunostimulant cytokine (IL-24) was demonstrated to be related to the activation of an apoptotic caspase cascade (primarily caspases-3 and -8) in HCC (37).

A future direction of cancer research is to find a new combination therapy with OAds to treat tumors. Conversely, CAR is vital for ensuring virus uptake, gene transfer and acts as a principal receptor for the coxsackie B virus and adenovirus. In summary, CAR expression could serve as a biomarker for studying and estimating the results of gene therapy, and increasing its expression may enhance cellular sensitivity to adenovirus infection. Therefore, there is a potential need to research new therapies and to detect the expression of the CAR, concurrently.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SW and XM conceived and designed the study. XY, SL, HW and WC performed the experiments. XY wrote the paper. HW, WC, XM and SW reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Ethics approval and consent to participate

The patient samples were collected from Zhejiang Provincial People's Hospital and all patients provided written informed consent. The study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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