Coxsackie Adenovirus Receptor Expression in Carcinomas of the Head and Neck

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Abstract. *Background: Advanced stage head and neck squamous cell carcinomas (HNSCC) have a poor prognosis, this being particularly true for undifferentiated carcinomas. Adenoviral oncolytic therapy, whose success depends on the expression of the coxsackie adenovirus receptor (CAR) on tumour cells, might be an interesting therapeutic option. Thus CAR expression in HNSCC was evaluated in the current study. Patients and Methods: CAR expression in 41 cases of HNSCC was investigated immunohistochemically. Results: CAR expression was very heterogeneous and was more abundant in well differentiated carcinomas than in less differentiated ones. Expression decreased from 72.4% in G1 tumours to 56% in G4 tumours. Conclusion: As CAR expression decreases during malignant progression in HNSCC, its down-regulation in advanced grades of HNSCC is potential indicator of tumour progression. With regard to oncolytic therapy, CAR expression analysis should be performed prior to adenoviral oncolytic treatment to stratify patients for treatment.*

Squamous cell carcinoma of the head and neck (HNSCC) represents about 6% of all cancers worldwide and is the sixth most common type of cancer (1). Out of these, nasopharyngeal cancer (NPC) accounts for 0.2% of all cancer in Central Europe and the United States, but is more common in Southeast Asia, Greenland and North Africa, where it is endemic. This type of cancer represents a particular clinical challenge as it is diagnosed at a late stage because of its initially non- specific and slight symptoms. In terms of radiosensitivity, tumour spread and survival rates, undifferentiated NPC differs in tumour biology from other carcinomas of the head and neck. This results in an overall poor prognosis with a median five-year survival of just 32.1% (2). Surgery is the treatment of choice (3) but most patients with an advanced stage die because of locoregional spreading (4). For this reason, combined radio- and chemotherapy is under investigation (3, 5).

Due to the generally poor prognosis, novel therapeutic approaches are urgently needed. One of the more promising approaches is oncolytic viral therapy in which tumour-specific viruses are generated which specifically lyse tumour cells. The oncolytic adenovirus ONYX- 015 is licensed in China, but regarding the treatment of head and neck cancer had a response rate of only 10% (6).

Viral binding to the cell and internalization into the cytoplasm is mediated by the coxsackie adenovirus receptor (CAR). The identification of CAR advanced the understanding over the adenoviral infection (7). CAR is a 46-kDa integral membrane protein which belongs to the immunoglobulin superfamily (8, 9). Although the details about the molecular structure and interaction between viruses and CAR are known (7), its cellular function and regulation remains enigmatic. The localization on cell to cell adhesive structures and on tight junctions assumes a potential role in formation and stabilization of cell to cell contacts (8). CAR expression is highly variable according to different tissues, developmental stages and pathologic conditions (10-12). Especially in malignant tissue, heterogenic expression was recognized (13), whereas increased expression in adenomas and metastatic lesions has been described (12). The positive correlation between the expression level of the primary receptor and the therapeutic efficacy (15) means that a low expression level of CAR leads to resistance against adenoviral infection (16-18).

To evaluate the potential efficacy of adenoviral oncolytic therapy for undifferentiated HNSCC, we examined the expression of CAR in solid head and neck carcinomas, with particular regard to NPC, from 41 patients.

Patients and Methods

Patients and samples. A total of 73 formalin-fixed, paraffin-embedded tissue samples from 41 patients with solid anaplastic HNSCC excised between 1983 and 1987, including 28 male and 13

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Key Words: Coxsackie adenovirus receptor (CAR), nasopharyngeal carcinoma, oncolytic therapy, immunohistochemistry.

Table I. *Patients' characteristics and tumour CAR expression.*

Specimen/case	Gender/Age	Loc.	Diag.	Grade	CAR	Specimen/case	Gender/Age	Loc.	Diag.	Grade	CAR
1/1	M/59	LN	UCNT	1	-	37/25	M/45	LN	SCC	1	2
2/2	M/42	LN	UCNT	3	1	38/25	M/45	LN	SCC	1	1
3/3	M/48	NP	LE	4	-	39/25	M/45	LN	SCC	1	-
4/4	M/75	LN	SCC	3	1	40/25	M/45	LN	SCC	1	1
5/4	M/75	LN	SCC	3	-	41/25	M/45	LN	SCC	1	-
6/4	M/75	LN	SCC	3	1	42/25	M/45	LN	SCC	1	1
7/5	M/60	LN	SCC	2	1	43/25	M/45	LN	SCC	1	-
8/6	M/42	NP	UC	4	-	44/26	M/56	NP	UC	4	2
9/7	F/45	TO	UC	4	1	45/26	M/56	NP	UC	4	-
10/7	F/45	TO	UC	4	1	46/26	M/56	NP	UC	4	-
11/8	M/38	OP	SCC	1	2	47/27	M/63	NP	UC	4	-
12/9	F/67	TG	UCNT	1	2	48/28	F/43	LN	NK	3	-
13/9	F/67	TG	UCNT	1	1	49/29	M/39	LN	UCNT	3	-
14/9	F/67	TG	UCNT	1	1	50/29	M/39	LN	UCNT	2	-
15/10	F/45	LN	UCNT	4	-	51/30	M/62	NP	SCC	1	1
16/11	M/43	LN	SCC	2	-	52/31	M/48	NP	SCC	1	1
17/11	M/43	LN	SCC	2	1	53/32	M/76	NP	SCC	1	1
18/11	M/43	LN	SCC	2	1	54/32	M/76	NP	SCC	1	-
19/12	M/uk	NP	UC	4	1	55/32	M/76	NP	SCC	1	1
20/13	M/40	NP	UC	4	1	56/33	M/37	OP	SCC	1	1
21/13	M/40	NP	UC	4	1	57/33	M/37	OP	SCC	1	2
22/14	M/81	NP	UC	4	2	58/33	M/37	OP	Hyp	0	1
23/15	F/44	LN	UCNT	4	1	59/34	F/39	LN	NK	1	1
24/16	M/47	LN	SCC	3	1	60/35	M/64	LN	SCC	2	1
25/17	F/66	LN	UC	4	-	61/35	M/64	LN	SCC	2	1
26/18	F/50	HP	SCC	3	2	62/36	M/74	LN	SCC	3	1
27/19	F/63	NP	NK	1	1	63/37	F/64	LN	UCNT	1	1
28/19	F63	NP	NK	1	1	64/38	F/61	LN	SCC	1	1
29/19	F/63	NP	NK	1	1	65/39	M/64	TO	SCC	1	-
30/19	F/63	NP	NK	1	-	66/40	F/62	TO	SCC	3	-
31/20	M/63	LN	UCNT	4	-	67/41	M/74	LN	UCNT	4	1
32/21	M/76	LN	UCNT	4	-	68/41	M/74	LN	UCNT	4	1
33/22	F/64	LN	UCNT	2	-	69/41	M/74	LN	UCNT	4	1
34/23	M/64	LN	UCNT	2	-	70/41	M/74	LN	UCNT	4	1
35/24	M/55	NP	UC	4	-	71/41	M/74	LN	UC	4	-
36/25	M/45	LN	SCC	1	-	72/41	M/74	LN	UC	4	1
						73/41	M/74	LN	UC	4	1

– indicates negativity of the immunohistochemical staining, 1 a CAR positive and 2 a strongly CAR positive staining. Loc, Localization; Diag, diagnosis; uk, unknown; LN, lymph node; NP, nasopharynx; TO, tonsil; OP, oropharynx; HP, hypopahrynx; TG, thyroid gland; UCNT, undifferentiated carcinoma of the nasopharyngeal type; UC, undifferentiated carcinoma; SCC, squamous cell carcinoma; NK, non-keratinizing; Hyp, hyperplasia. Age is given in years.

female patients, were obtained from the Department of Oral and Maxillofacial Surgery, University Hospital Hamburg-Eppendorf, Germany. The mean age of patients was 55.93±12.61 years (range: 37-81 years). Each tumour was classified according to the American Joint Committee on Cancer as G1, well differentiated; G2, intermediately differentiated; G3, poorly differentiated; and G4, undifferentiated tumours (19). The characteristics of the population under study are summarized in Table I.

Immunohistochemistry. Tissue sections of 5 μm thick sections were deparaffinized and rehydrated in xylene and in series of graded ethanol and finally placed in 10 M citrate buffer (pH 6.0) for heat-induced antigen retrieval (initially 3 min at 1000 W, then three times for 5 min at 500 W in a commercial microwave oven). Subsequently, slides cooled for 20 min and were then transferred to a cold buffer. After 10 min, slides were transferred to Tris-buffered saline (TBS, pH 7.6) for 5 min, which was again changed twice to fresh TBS for 5 min each. The sections were blocked in 10% normal swine serum/DAKO® Antibody Diluent with Background Reducing Components (DAKO Corp., Carpentaria, CA, USA) for 30 min at room temperature and thereupon incubated in primary antibody Anti-CxADR (diluted 1:200; Sigma Aldrich, St. Louis, MO, USA) overnight at 4˚C. Sections were then washed in TBS and incubated in secondary biotinylated swine anti-rabbit immunoglobulin (diluted 1:200; DakoCytomation, Glostrup, Denmark) for 30 min followed by a washing step and an incubation with an ABC alkaline phosphatase kit (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min. After the last washing step with TBS, slides were

transferred in the visualizing mixture containing Naphthol-AS-bisphosphate and hexatozised New Fuchsin to visualize the enzyme reactivity of the alkaline phosphate complex. The reaction was carried out in the dark and was stopped after 30 min under running tap water for 5 min, followed by counterstaining with Mayer's hemalaun diluted 1:1 in distilled water for 3 s, blued under running tap water and then after dehydration in series of increasing ethanol concentrations, sections were mounted with Eukitt (Kindler, Freiburg, Germany).

Negative and isotype controls were treated the same way with either pure DAKO® Antibody Diluent with Background Reducing Components or with rabbit immunoglobulin normal fraction (DAKO A/S, Glostrup, Denmark) instead of CAR polyclonal primary antibody. Cells in agar from OH1 small cell lung cancer cell line (supplier detail) which had previously been proven to express the receptor abundantly was used as an appropriate positive control.

Evaluation of Immunohistochemistry. All tissue sections were judged by their percentage of CAR-expressing cells and the intensity of the staining. Specimens were considered as positive when more than 5% of the tumour cells were stained. The scoring was 0=0-4%, 1=5-49% and 2=50-100% for percentage of cells stained and the intensity was scored as 0=no staining, 1=weak staining, 2=moderate staining and 3=intense staining. Finally, the immunoreactivity was graduated by multiplication of the intensity and percentage as 0=negative; 1=scores 1 and 2, weak staining; and 2=scores 3-6, strong staining. Results were estimated by two independent evaluators. Cases with differing evaluation were resolved by reviewing and a consensus was reached.

Statistical analysis. Associations of the grade and CAR immunoreactivity were assessed using the Chi-square test. Statistical analyses were performed using the software package SPSS for Windows (Version 19; SPSS Inc., Chicago, IL, USA).

Results

In this study, CAR protein expression was evaluated in undifferentiated head and neck caarcinomas and NPC. The staining results depending on the grade of differentiation of the carcinoma are summarized in Table II.

CAR expression was found in normal tissue in a localization which is characterized by its cell junctions. The intense staining of the *stratum germinativum* of the epithelium, as well as the staining of the *serous acinus* and *striated ducts* of the salivary glands, was very intense (Figure 1). All cells in the one case of hyperplasia were CAR positive. In general, the intratumoural distribution of CAR expression was very heterogeneous. Furthermore, the expression of CAR immunoreactivity varied between several biopsies from the same patient, and between patients having the same G status. From all 29 G1 tumours, 21 (72.4%) were positive, out of these four (13.8%) showed strong CAR staining; eight cases (27.6%) were CAR negative (Figure 2). The percentage of CAR-positive tumours in the group of G2 tumours was even lower, with five cases (55.6%) with only weak CAR positivity. The results for G3 were similar, with six (60.0%) positive carcinomas, including one sample with high CAR expression. Fourteen tumours (56.0%) of all grade 4 tumours were positive, two of which (8.0%) were strongly stained. Thus, the highest proportion of positive tumours was found in G1 tumours and the percentage of stronger positive staining decreased from G1 to G4 (Figure 3). However, none of these correlates were statistically significant.

Interestingly, we found a lymphatic vessel penetrated by tumour cells derived from a metastasis of the jaw angle from a solid squamous cell carcinoma (SCC) grade 2 which was also CAR positive (Figure 4).

Table II. *Summary of the CAR immunoreactivity in association with the tumour grade of solid anaplastic head and neck carcinomas. CAR expression decreases with higher grading.*

Variable	Number (%)	CAR expression score		
		-	1	2
N=74		29 (36.2)	44 (55.0)	7 (8.8)
Gender				
Male	28 (68.3)	11 (39.2)	13 (46.4)	4 (14.3)
Female	13 (31.7)	5 (38.5)	6 (46.2)	2 (15.4)
Age (mean±SD, years)	55.925±12.613			
Grade				
Hyperplasia	1 (1.4)	0	1 (100.0)	0
G1	29 (39.2)	8 (27.6)	17 (58.6)	4 (13.8)
G2	9 (12.2)	4 (44.4)	5 (55.6)	0
G3	10 (13.5)	4 (40.0)	5 (50.0)	1 (10.0)
G4	25 (33.8)	11 (44.0)	12 (48.0)	2 (8.0)
		Negative	Positive	
Grade				
1=0+1	30 (40.5)	8 (26.7)	22 (73.3)	
2=2+3	19 (25.7)	8 (42.1)	11 (57.9)	
3=4	25 (33.8)	11 (44.0)	14 (56.0)	

Discussion

Head and neck cancer, especially the type affecting the nasopharyngeal region, is an insidious neoplasm because of its initial slight and nonspecific clinical symptoms and a high potential for distant metastasis. Most patients are diagnosed when the tumour has already reached advanced stage (19). High grade of malignancy, as well as advanced tumour stage, leads to an unfavorable prognosis. Surgery and/or radiation are the therapy of choice but, unfortunately, the 5-year survival rates have shown no improvement over the last two decades. Although NPC is highly radiosensitive, radiation therapy is limited by the often close localization of the tumour to dose-limiting organs such as the brain, eye or ear. A combination with chemotherapy increases survival (20), however, new therapeutic options are needed due to the poor

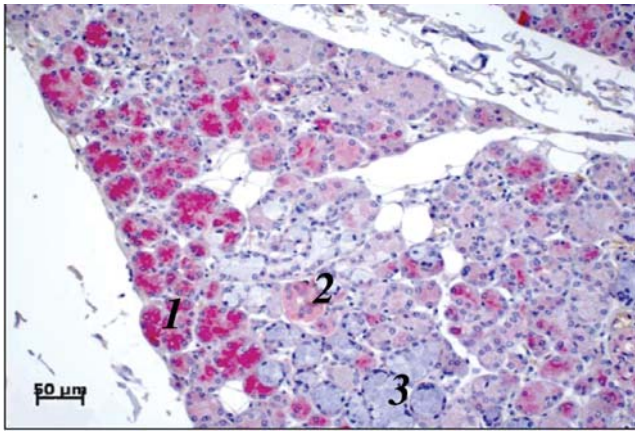

Figure 1. *Immunohistochemistry of a salivary gland using the polyclonal anti-CXADR antibody. The high CAR expression of the serous acini (1) in the salivary gland stands out. The striated duct (2) is CAR positive as well, while the mucinous acini (3) are negative for CAR expression.*

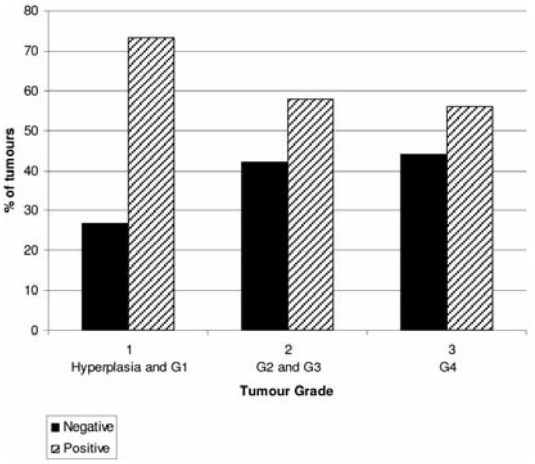

Figure 3. *Statistical evaluation of CAR-positive staining according to the grading of the tumours. 1 includes hyperplasia and well-differentiated carcinomas (G1); 2 intermediate (G2) and poorly differentiated carcinomas (G3); and 3, undifferentiated carcinomas (G4). CAR expression decreases with advancing grade of differentiation.*

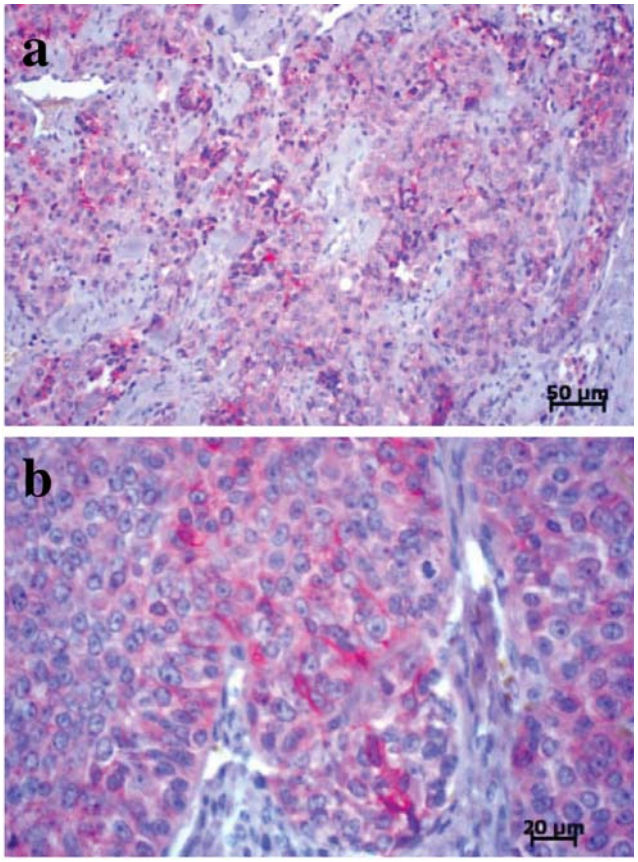

Figure 2. *Immunohistochemistry of nasopharyngeal carcinoma using the polyclonal anti-CXADR antibody. a: The well-differentiated (G1) tumour cells exhibit a high CAR expression (80%, positive cells intensity of 3). b: In the high-power magnification, the intensely labelled cell membranes can be seen.*

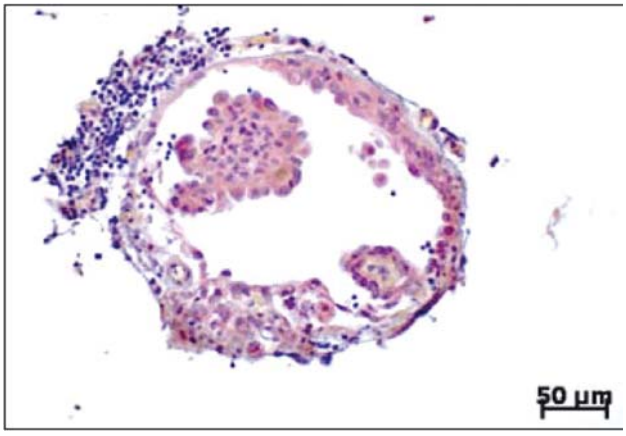

Figure 4. *Immunohistochemistry of a metastasis using the polyclonal anti-CXADR antibody. Intraluminal metastatic cells from a solid G2 tumour in a lymphatic vessel. All malignant cells were CAR positive.*

prognosis. A new possible therapy option could be treatment with a replication selective oncolytic virus.

The present study was therefore undertaken to investigate if the presence of the CAR protein in tumour cells makes adenoviral treatment for carcinomas of the oro- and nasopharyngeal region feasible, as adenoviral infection strongly depends on the CAR expression of the cells to be infected. The immunohistochemical staining showed a heterogeneous CAR expression pattern in the clinical specimens. Intratumourally and interindividually the CAR

expression was heterogeneous. These results match with observations made in clinical samples of osteosarcomas, Ewing's sarcomas (21) and thyroid tumours (22). Pan *et al*. (23) in a 6-year follow-up showed that a combination of a recombinant adenovirus-p53 and radiotherapy can increase the 5-year overall survival rate by 7.5%. Even *in vitro* in head and neck cancer cell lines, including one pharyngeal carcinoma cell line, the receptor density on the cell surface varied significantly between the evaluated cell lines, whereby the number of adenovirus receptors correlated to the susceptibility to the adenovirus (24). This heterogeneity could limit infection, but tumours which express CAR at a high level would qualify for an oncolytic therapy. Prior to oncolytic adenovirus therapy, evaluation of the CAR expression level would be useful to enhance chances of success.

Because of its transmembrane localization and association with tight junctions, it has been assumed that a correlation exists between grading and CAR expression. Hence here, HNSCCs were classified according to their grade. The classification indeed showed differences in CAR expression level in different grades. G1 tumours had the highest proportion of positive tumour cells (72.4%) while positive staining decreased with loss of dedifferentiation (differentiation grades: 1=73.33%, 2=57.9%, 3=56% positive tumours). Although the results were not statistically significant, which may be due to the small number of cases, there is a visible trend of the correlation between differentiation and CAR expression.

Jee *et al*. (25) showed that CAR expression in normal epithelium is much higher than in malignant tissues of HNSCC. Our results corroborated with these findings. Normal epithelial cells exhibited strong staining in typical apical localizations. First of all, the CAR protein was found in the *stratum germinativum* of the epithelium. This is characterized by strong desmosomes and adherens junctions who mediate intercellular connections, as well as hemidesmosomes connecting the basal lamina. Aside from that localization, epithelial cells at the luminal side of the *serous acinus* of the submandibular gland were strongly CAR positive. Furthermore, membranous CAR localization was detected at the cell boundaries of the *striated duct* epithelia.

Although the physiological and biological function of CAR is not definitively known, an association with tight junctions is attributed to it (26). It can be assumed that CAR plays a role in the formation and stabilization of cell to cell contacts. The *stratum basale*, as well as the serous acini and *striated ducts* of the salivary gland, are characterized by distinct cell contacts which confer a cell polarity on them. These characteristics of epithelium are lost during tumour progression. Our results show that the tight junction-associated protein CAR is especially down regulated in high-grade carcinomas. A down-regulation of CAR in the primary tumour could increase the tendency for cells to detach from the initial tumour and the development of distant metastases. A possible explanation for the down-regulation of CAR in higher graded head and neck carcinomas could be that there are regulatory mechanisms of CAR expression which are associated with the process of de-differentiation. In more differentiated carcinomas, epithelial cells show specific characteristics, particularly because of their cell adhesion contacts. During tumour progression, cell junctions are lost and cells become more mobile and invasive. Likewise, such a down-regulation was found for epithelial glycoprotein-2 (27) and similarly, the loss of CAR expression could indicate a higher migratory potential. This hypothesis is underlined by the different degrees of histological differentiation of NPC. They are classified as keratinizing squamous carcinoma and non- keratinizing squamous carcinoma, which are subdivided into a differentiated and an undifferentiated type. This classification is not only applicable for epidemiological research but also significant for the prognosis of the patient. In particular undifferentiated NPCs have a high tendency to metastasize, resulting in a clinically highly malignant neoplasm with a poor prognosis. These carcinomas, also known as Schmincke-Regaudtumour, are classified as G4 in the grading scale, the grade with the smallest part of tumours with high CAR expression.

With regard to a therapeutic option different conclusions can be drawn. Low response rates reported in clinical studies (6, 28) may, in part, be due to patient selection. No determination of CAR expression is usually performed prior to the initiation of the treatment and hence lack of patient selection could explain the relatively low response rate to this treatment. Because of the heterogeneous expression of CAR protein, its level of expression should be evaluated prior to commencement of an oncolytic adenoviral therapy. Based on our immunohistochemical staining results, better differentiated tumours are more promising targets for oncolytic therapy. Head and neck carcinomas classified as G1, in particular, seem to be appropriate for oncolytic therapy because of the high proportion of tumours expressing CAR and, thus, a higher rate of infection with the oncolytic virus can be achieved on the tumour cells.

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