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Allogenic antibody-mediated identification of head and neck cancer antigens

Jens Rauch[a,1], Martin Ahlemann[a,1], Martina Schaffrik[b,c,1], Brigitte Mack[a], Suna Ertongur[a], Michaela Andratschke[a], Reinhard Zeidler[d], Stephan Lang[a], Olivier Gires[a,b,c,*]

[a] *Department of Head and Neck Surgery, Ludwig-Maximilians-Universität München, Marchioninistr.15, D-81377 Munich, Germany*
[b] *Clinical Cooperation Group Molecular Oncology, Department of Head and Neck Research, Ludwig-Maximilians-Universität München, Marchioninistr.15, D-81377 Munich, Germany*
[c] *GSF-National Research Center for Environment and Health, Marchioninistr.25, D-81377 Munich, Germany*
[d] *Vaecgene Biotech Inc., Marchioninistr. 25, D-81377 Munich, Germany*

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Abstract

Recently, we described a new target-identification technology, autoantibody-mediated identification of antigens (AMIDA). AMIDA takes advantage of autologous serum autoantibodies to identify disease-associated antigens. Here, we evaluated the allogenic variant of AMIDA (allo-AMIDA), using permanent cancer cell lines as an antigen-pool rather than primary biopsy samples. Twelve different proteins were retrieved exclusively with antibodies from cancer patients, but not from healthy donors. The expression of three of these antigens, e-FABP, hnRNP H, and Grb2, was evaluated in more detail. All three proteins were strongly overexpressed in primary carcinomas and metastases thereof, as compared to healthy epithelium. Additionally, serum reactivity against e-FABP was detected in 20% of cancer patients but only 2% of healthy volunteers. In summary, we demonstrate that permanent cancer cell lines represent a reliable source for tumour-associated antigens. Moreover, we show that allo-AMIDA is suitable for the identification of tumour-specific antigens overcoming the limitations of autologous screening techniques.

Keywords: AMIDA; e-FABP; hnRNP H; Grb2; Bio-Plex; Serum antibodies

Carcinomas of the upper aerodigestive tract are among the most common types of cancer [1]. The five-year survival rate of patients with head and neck squamous cell carcinomas (HNSCCs) has not significantly improved throughout the last decades, despite major progresses in surgical techniques and chemotherapy [2]. Occult disseminated tumour cells often resist chemotherapy and are the origin for the so-called minimal residual disease (MRD), and thus a major cause of relapse in cancer patients [3]. Much emphasis has been put into the identification of new molecular targets for immunotherapeutic approaches and for the early detection of tumours. In this respect, it is of help that tumours elicit multiple specific immune responses in the host including the generation of antibodies specific for tumour proteins, which represent an 'immunological tumour fingerprint.' These antibodies are valuable tools for the identification of tumour-associated antigens (TAAs), which are indispensable for new therapeutic approaches. Furthermore, TAA-specific antibodies may themselves be used as circulating tumour markers for diagnostic purposes.

* Corresponding author. Fax: +49 89 7095 6896.
E-mail address: ogires@hno.med.uni-muenchen.de (O. Gires).
[1] These authors contributed equally.

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We have taken advantage of cancer-specific serum antibodies to develop a new technology for the identification of disease-specific molecules, coined AMIDA. AMIDA is based on the initial immunoprecipitation of TAAs with serum antibodies, followed by 2D-PAGE separation and mass spectrometry analysis [4]. Here, we describe a variant allogenic form of AMIDA (allo-AMIDA) using permanent human cancer cell lines as a protein source in order to overcome the limitation of biopsy availability and heterogeneity. Twelve proteins were identified that exclusively precipitated with antibodies from cancer patients but not from healthy controls. The in-depth analysis of the expression patterns of three of these proteins revealed a strong overexpression in carcinomas and metastases. Also, we demonstrate that antibodies specific for one allo-AMIDA antigen (e-FABP) were significantly elevated in 20% of cancer patients but only in 2% of healthy donors, making these antibodies a potential circulating tumour marker.

Materials and methods

Cell lines and lysate preparation. FaDu cells (ATCC HTB-43) and PCI-1 cells (a kind gift from Prof. Whiteside) derived from human HNSCC were propagated under standard conditions. Whole cell extracts were obtained by resolving cell pellets in lysis buffer [TBS, pH 7.4, 1% (vv^{-1}) Triton 100 (Merck, Darmstadt, Germany), protease inhibitor cocktail Complete (Roche Diagnostics, Mannheim, Germany)]. After rocking the suspension for 45 min at 4 °C, the insoluble particulate fraction was removed by centrifugation (10 min at 4500*g*, 4 °C). The protein concentration was determined using a BCA Protein Assay Reagent Kit according to the manufacturer's instructions (PIERCE, Rockford, US).

Serum samples and antibody purification. Blood samples from eight HNSCC patients were taken after informed consent. Venous blood (20 ml) was centrifuged at 1700*g* for 15 min and the serum was stored at −20 °C. Antibody purification was performed using Montage spin columns containing Protein G affinity media (Millipore, Billerica, US) according to the manufacturer's protocol. Antibodies were adjusted to a concentration of 10 mg ml^{-1} in PBS supplemented with 0.1% (wv^{-1}) sodium azide and stored at 4 °C.

Immunoprecipitation. All centrifugation steps were carried out for 5 min at 500*g* at room temperature unless stated otherwise. For immunoprecipitation, 500 μl of a protein G bead suspension (Amersham Biosciences, Freiburg, Germany) was incubated with 20 mg purified IgGs from cancer patients and, in parallel, a pool of human IgGs (100 healthy donors, Sigma–Aldrich, Lot 011K9019, Taufkirchen, Germany) for 30 min at room temperature. Beads were harvested by centrifugation and washed once with PBS, pH 7.4, and once with 200 mM triethanolamine, pH 8.2 (Sigma–Aldrich, Taufkirchen, Germany). Covalent cross-linking was performed in 200 mM triethanolamine, pH 8.2, containing 100 mM dimethylpimelimidate (Sigma–Aldrich, Taufkirchen, Germany). The cross-linking reaction was carried out for 45 min at room temperature under gentle shaking. Beads were washed once with 100 mM ethanolamine, pH 8.2, for 5 min, once with TBS, pH 7.4, and once with lysis buffer at 4 °C before the cell lysate of 1×10^8 cells was added. The suspension was incubated overnight at 4 °C. In order to remove detergents, salts, and unspecifically bound proteins, the beads were centrifuged, washed twice with lysis buffer and once with 50 mM Tris, pH 7.4 (Sigma–Aldrich, Taufkirchen, Germany). Beads were harvested by centrifugation and immunoprecipitated proteins were eluted by incubation in 470 μl of 2D lysis buffer (9 M urea, Amersham Biosciences, Freiburg, Germany; 4% wv^{-1} Chaps, Sigma–Aldrich, Taufkirchen, Germany; and 2.5 mM EDTA, Merck, Darmstadt, Germany) for 10 min, at room temperature, with constant rotation. Supernatants were obtained by centrifugation at 7000*g* at room temperature for 5 min. DTE (1% wv^{-1}, Sigma–Aldrich, Taufkirchen, Germany) was added after elution of the proteins from the Sepharose beads in order to avoid the premature release of IgG light chain due to disulfide bond reduction.

2D-PAGE and protein identification. Prior to isoelectric focusing, 0.5% vv^{-1} IPG-buffer, pH 4–7 (Amersham Biosciences, Freiburg, Germany), with 0.01% bromphenol blue (SERVA, Heidelberg, Germany) was added to the immunoprecipitated proteins. Samples were focused isoelectrically (IPGphor system, Amersham Biosciences, Freiburg, Germany, 80 kVh) using pH 4–7 24 cm IPG strips (Amersham Biosciences, Freiburg, Germany). Thereafter, proteins were separated according to their molecular weight on a vertical 11% SDS gel electrophoresis using the Ettan Dalt II separation unit (Amersham Biosciences, Freiburg, Germany).

Proteins separated by 2D-PAGE were visualized by MS-compatible silver staining [5]. Gels were scanned using the ImageScanner system (Amersham Biosciences, Freiburg, Germany) and spot patterns, analyzed by ImageMaster Elite 2D software. Proteins that precipitated exclusively with serum IgGs from cancer patients were excised from the gel using disposable scalpels. Excised protein spots were digested using Montage In-Gel digest (96) kit (Millipore, Billerica, US) according to the manufacturer's instructions. The peptide mixtures were concentrated in a Speed-Vac and desalted using μC18-ZipTips (Millipore, Billerica, US) as directed by the manufacturer. The purified peptide digest was eluted from the ZipTips using 2 μl matrix α-cyano-4-hydroxycinnamic acid (Bruker Daltonik, Bremen, Germany) prepared as a saturated solution in 0.1% vv^{-1} TFA/50% vv^{-1} acetonitrile, and 1 μl was loaded on a matrix assisted laser desorption ionization (MALDI) sample plate. Peptide mass fingerprinting analysis was performed on a Bruker Reflex III MALDI-TOF MS (Bruker Daltonik, Bremen, Germany). After removal of peptide masses resulting from keratin contamination and trypsin autoproteolysis, the corrected list of peptide masses was aligned with the MASCOT search protein database (http://www.matrixscience.com). Standard search parameters were used-database: MSDB, taxonomy: *Homo sapiens*, enzyme: trypsin, fixed modifications: carbamidomethyl, variable modifications: oxidation (M), and peptide tolerance: ±100 ppm. RMS errors for all proteins identified were ⩽45 ppm.

Immunohistochemistry. Specimens of squamous cell carcinoma and healthy mucosa were obtained from patients undergoing routine surgery. Briefly, tissues were shock frozen in liquid nitrogen and cut into 4 μm thick sections. For immunohistological staining polyclonal rabbit anti-human e-FABP antibody [6], polyclonal rabbit anti-human hnRNP H antibody (Bethyl Laboratories, Montgomery, US), and monoclonal mouse anti-human Grb2 antibody (BD Biosciences, San Diego, US) were used. Antigen–antibody complexes were visualized using the avidin–biotin–PO (ABC) method as previously described [7].

Quantification of antibody titers. Recombinant gluthathione *S*-transferase (GST) and human full-length GST–e-FABP fusion protein were expressed in *E. coli* using the pETM30 vector (EMBL, Heidelberg, Germany). Beadlyte glutathione beads (Biomol, Hamburg, Germany) were loaded with the recombinant proteins to saturation according to the manufacturer's instruction. Serum reactivities from healthy volunteers (normal human sera, NHS) and cancer patients were tested by incubation with GST or GST–e-FABP loaded GSH-beads (1 h, RT, 1:100 dil.) and subsequent detection using PE-coupled anti-human IgG antibodies (30 min, RT). The measurement of PE-fluorescence was performed in a Bioplex system (Bio-Rad, Hercules, US). The PE-fluorescence of the individual samples was compared for 50 labeled beads of each sample, and expressed as mean fluorescence intensities (MFI). Significance was calculated using the unpaired

Student's t test. A positive reaction was defined as a value above the mean value of NHS plus three standard deviations (cutpoint) [8]. Sensitivity (s) was determined as follows: $s = a:(a + b)$ where a is the number of patients' sera above cutpoint and b below cutpoint. Specificity is the percentage of healthy donor sera below cutpoint.

Results

Allo-AMIDA screening of HNSCC patients

In the present study, we have applied the AMIDA technology in a modified, allogenic setting to identify potential TAAs from head and neck carcinoma cell lines. Eight serum samples from patients with diagnosed HNSCCs were included in the allo-AMIDA screen performed as depicted in Fig. 1.

In-depth comparison of the obtained 2D-protein patterns, i.e., immunoprecipitates from tumour versus healthy serum, resulted in the identification of an average of 5–6 spots that precipitated exclusively with antibodies from cancer patients. Representative examples of differential spots are shown in Fig. 2. All differential spots were excised and subjected to MALDI-TOF mass spectrometry analysis. The resulting peptide mass fingerprints served to screen protein databases for the corresponding protein identity. Twenty-seven protein spots were identified with significant Mowse scores ($p \leqslant 0.05$). Four of them were immunoglobulin heavy or light chains and were not considered as potential TAAs. Furthermore, a subset of proteins (Grb2, hnRNP H, TRIP-1, PSP-1, and e-FABP) was identified repeatedly with sera of two or three HNSCC patients. In total, twelve different proteins were immunoprecipitated exclusively by antibodies derived from cancer patients, and thus represented potential TAAs (Table 1). The antigens identified were nuclear proteins (paraspeckle protein 1, hnRNP H), proteins involved in signalling (Grb2, TRIP1) and primarily in metabolism (e-FABP, cytidine deaminase), chaperones (Hsp27, BiP), and cytoskeletal proteins (TPMsk3, profilin II, cortactin, and tubulin β).

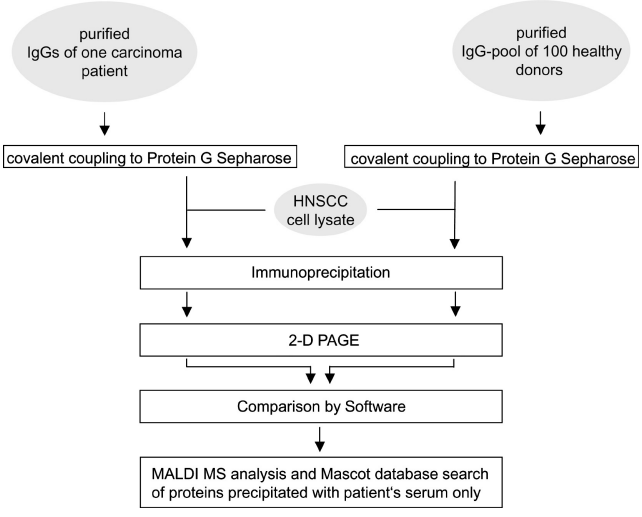

Fig. 1. Flow chart summary of the allo-AMIDA technique used to identify potential tumour antigens.

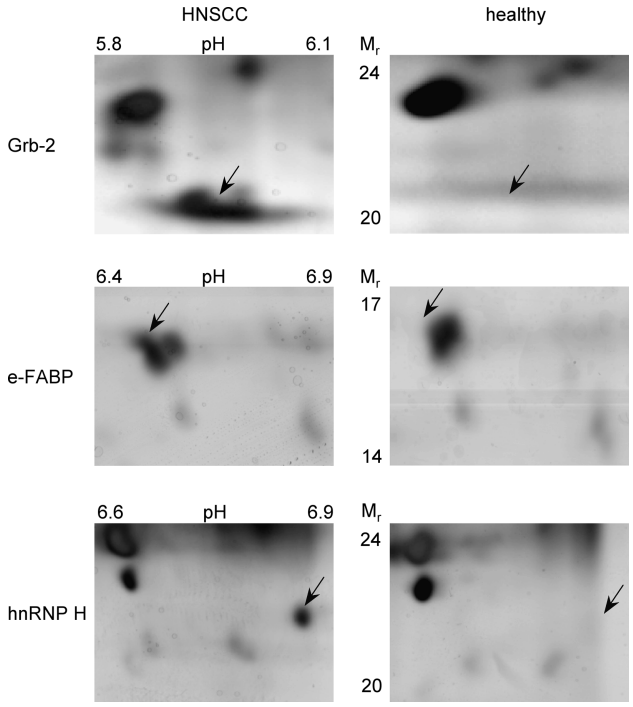

Fig. 2. Shown are 2D images of immunoprecipitated FaDu proteins using IgGs from healthy donors (right panel) and patients suffering from HNSCC (left panel). The proteins were separated by 2D-PAGE using a pH 4–7 IPG strip and a 11% SDS–polyacrylamide gel. Gels were stained with silver and imaged using the ImageMaster software.

Grb2, e-FABP, and hnRNP H are overexpressed in primary HNSCCs

Next, the expression levels of three proteins repeatedly identified (Grb2, hnRNP H, and e-FABP) were analyzed by immunohistochemistry using commercially available antibodies. Stainings were performed in a series of surgically removed HNSCC lesions and in healthy pharyngeal mucosa. HNSCCs as well as lymph node metastases showed a very strong staining for e-FABP in the majority of cases, whereas healthy epithelium expressed very low amounts (Figs. 3A–C). In total, three healthy mucosas of the head and neck area, 13 HNSCC lesions (larynx and pharynx), and 5 lymph node metastases were subjected to immunohistochemical analysis. An overexpression of e-FABP was detected in 69% of the pharyngeal and laryngeal lesions as compared to healthy epithelium, where e-FABP was consistently weakly expressed (Table 2).

The second protein analyzed, i.e., Grb2, was faintly detectable in cells of the basal membrane in intact, healthy epithelium, but was lacking in cells of suprabasal layers. In contrast, tumour cells of primary biopsies

Table 1
Proteins identified as targets of the humoral response in HNSCC by allo-AMIDA

Prot	Mascot search result	SwissProt Accession number	Score	Sequence coverage (%)	RMS error (ppm)	Theor. MW (kDa)	Exp. MW (kDa)	Theor. PI (pH)	Exp. PI (pH)	Frequency	
										FaDu n/t	PCI-1 n/t
1	e-FABP	Q01469	125	70	18	15.3	16	6.54	6.5	2/8	3/5
2	Cytidine deaminase	P32320	86	61	17	16.6	17	6.55	6.3	0/8	1/5
3	Grb 2	P29354	122	51	19	21.5	22	7.19	6.0	2/8	0/5
4	TRIP-1	Q13347	168	50	35	36.9	36	5.38	5.6	2/8	0/5
5	PSP1, α	Q8WXF1	186	26	44	58.8	50	6.26	6.3	3/8	0/5
6	hnRNP H	P31943	107	25	30	49.5	23	5.89	6.8	2/8	0/5
7	Tropomyosin 3 isoform 2	Q8TCG3	67	28	45	28.8	27	4.72	4.8	1/8	0/5
8	Profilin II	P35080	108	41	12	15.0	16	6.75	5.6	1/8	0/5
9	Cortactin	Q14247	96	17	41	61.8	66	5.24	5.6	1/8	0/5
10	Tubulin β1	P07437	132	33	29	49.3	38	4.75	5.5	1/8	1/5
11	BiP protein	Q9UK02	79	18	34	72.1	70	5.03	5.0	1/8	0/5
12	Hsp27	P04792	165	42	20	22.4	24	7.80	6.9	1/8	1/5

n, number of reactive patients' sera to the tumour antigen detected in different screens; *t*, total number of patients' sera used in AMIDA screens; and RMS error, root mean square error, indicates standard deviation in ppm for matched peptides as compared to theoretical values.

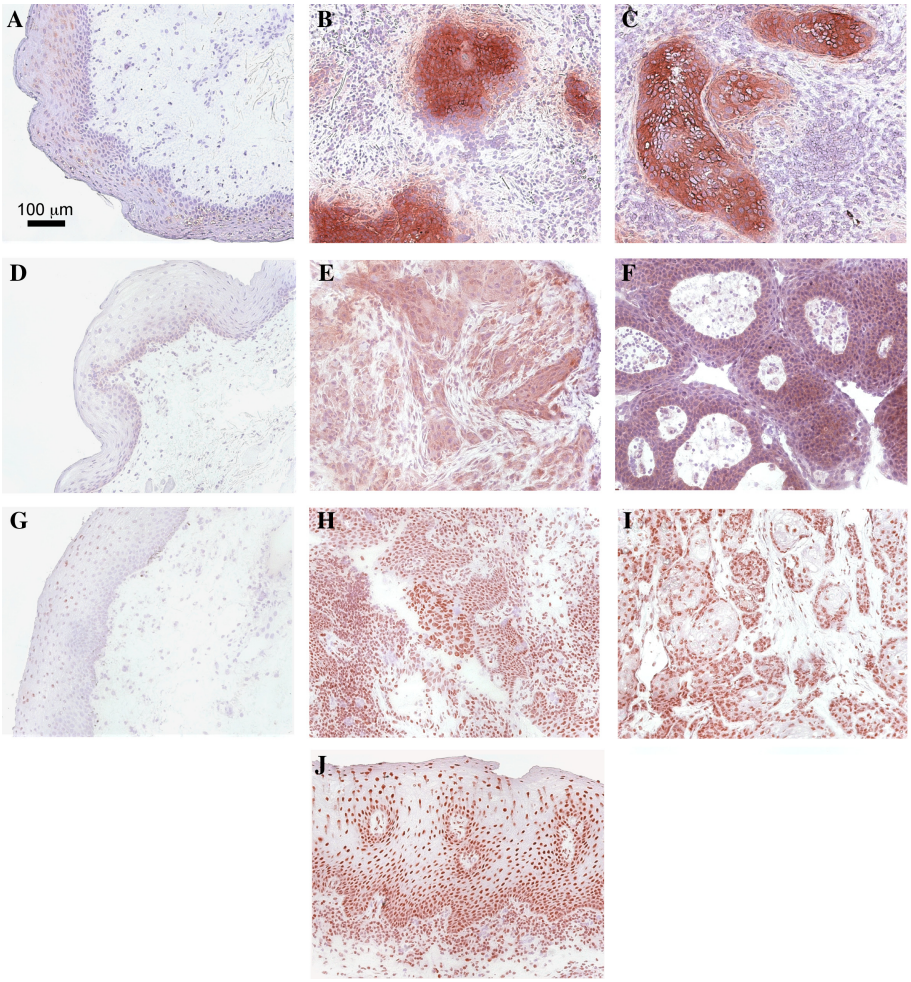

Fig. 3. Immunohistochemical staining of e-FABP (A–C), Grb2 (D–F), and hnRNP H (G–J) in healthy mucosa (A, D, and G), head and neck carcinoma tissues (B, E, and H), lymph node metastases (C, F, and I) and hyperplasia (J).

Table 2
Expression pattern of e-FABP in HNSCC

Localization	Expression pattern			
	–	+	++	+++
Pharynx	0/11 (0%)	4/11 (36%)	2/11 (18%)	5/11 (46%)
Larynx	0/2 (0%)	0/2 (0%)	1/2 (50%)	1/2 (50%)
Metastasis	0/5 (0%)	0/5 (0%)	1/5 (20%)	4/5 (80%)
Healthy mucosa	0/3 (0%)	3/3 (100%)	0/3 (0%)	0/3 (0%)

+++, strong expression; ++, moderate expression; +, weak expression; and –, no expression.

displayed a robust expression of the protein independently of the cell layer examined (Figs. 3D and E). In lymph node metastases the extent of Grb2 overexpression was even more conspicuous and restricted to the tumour cell mass (Fig. 3F).

The expression of hnRNP H increased at the transition from histologically healthy to hyperplastic and tumour tissue (Figs. 3G–J). HnRNP H was expressed only in cells of the basal membrane layer of healthy epithelium, whilst a robust expression was observed in tumour cells and metastases (Figs. 3H–J). The observed overexpression clearly resulted from a transcriptional up-regulation and not protein stabilization, as assessed by in situ hybridization experiments (data not shown). Thus, e-FABP, Grb2, and hnRNP H proteins were overexpressed in primary HNSCCs as compared to healthy tissue samples.

E-FABP elicits a humoral response in HNSCC patients

The isolation of e-FABP with serum antibodies from three out of eight HNSCC patients prompted us to assess the frequency and titer of antibodies to e-FABP in a larger set of cancer patients and healthy donors. For this purpose, we used an adapted Bioplex system combining the principle of an ELISA with the Luminex fluorescent-bead-based technology [9]. The reactivity of sera from healthy donors ($n = 48$) or HNSCC patients ($n = 59$) against a GST–e-FABP fusion protein or GST alone was determined in a Bioplex device. The specific e-FABP reactivity was calculated as the difference of the reactivity to GST–e-FABP and GST alone. Serum reactivities were scored positive when exceeding the cut-point (MFI 5950, see Materials and methods). Only one out of 48 normal human sera, but 12 out of 59 HNSCC patients' sera tested positive (Fig. 4), which results in a calculated sensitivity of 20.3% and a specificity of 97.9%.

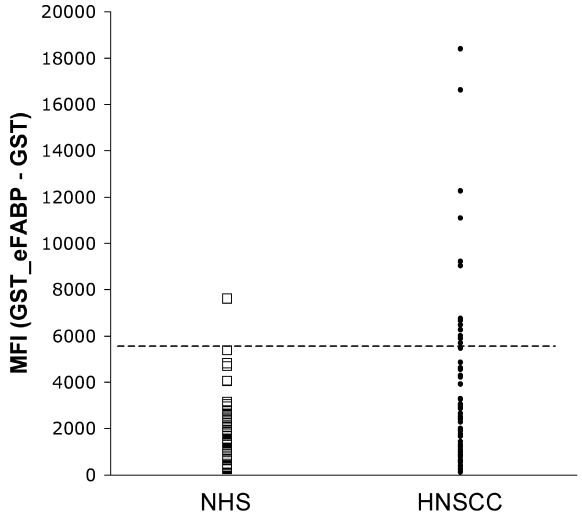

Fig. 4. Survey of anti-e-FABP antibodies in the sera of cancer patients. Reactivity of sera from 48 healthy donors (normal human sera, NHS) and 59 HNSCC patients was tested against GST–e-FABP and GST recombinant protein. e-FABP specific MFI was calculated as the difference of GST–e-FABP fusion protein and GST. A positive reaction is defined as an MFI value of a serum that exceeds the mean MFI value of NHS by 3 SD values (dashed line). (Significance was calculated with an unpaired Student's t test, and with $p < 0.005$, was found to be extremely significant).

Discussion

Sensitive and specific molecular tumour markers for the early detection of cancer are rare, with a few exceptions such as the prostate specific antigen (PSA). Since early detection of cancer significantly improves the clinical outcome for patients, the search for new molecular markers has become a challenging focus for basic and clinical research. Serological screening techniques such as SEREX [10], SPEAR, SERPA, and PROTEOMEX are hitherto promising experimental approaches, which resulted in an increased understanding of tumour immunology and uncovered the existence of novel tumour antigens [11]. AMIDA is a novel technology based on the immunoprecipitation of tumour-specific antigens using immobilized serum antibodies together with primary carcinoma biopsies, in an autologous setting. This immunoprecipitation proved to be a selective and efficient biological filter in order to reduce the complexity of the protein mix to be separated upon 2D-PAGE [4]. However, autologous screening techniques have limitations: (i) the yield of tumour cells from primary biopsies is a limiting factor and (ii) tumours are of heterogeneous

composition and contain varying numbers of non-malignant infiltrating cells. In order to circumvent these limitations, we set up allo-AMIDA using purified immunoglobulins from carcinoma patients along with permanent cell lines, which represent a homogeneous and unlimited source of proteins. As a consequence, patients' sera can be screened in a repetitive manner under standardized conditions. In healthy subjects, serum reactivities mainly originate from allogenic cross-reactions, the presence of multiple natural autoantibodies [12], and non-specific protein interactions. We have overcome this issue by the use of pooled immunoglobulins from healthy donors as a very stringent control. Furthermore, in the allo-AMIDA setting, all potential tumour antigens were isolated from cell lines. The detailed analysis of these antigens revealed their tumour-specific overexpression in vivo, too. Thus, carcinoma cell lines represent a valid protein pool, confirming the rationale of allo-AMIDA.

Some of the proteins identified have already been described previously as humoral antigens in cancer patients. Anti-Hsp27 antibody reactivity was observed in sera from patients with renal cell carcinoma [13] and breast cancer, and was associated with an improved survival [14]. In contrast, the presence of BiP-specific antibodies correlated with progression and shorter overall survival in patients with prostate cancer [15]. Lagarkova et al. [16] identified cortactin in a SEREX analysis of breast carcinomas and demonstrated a specific humoral response in HNSCC patients.

In order to induce cellular and humoral immune reactions, antigens have to be expressed at levels sufficient for cross-presentation by mature professional antigen-presenting cells [17]. This is in line with our own data concerning the overexpression of Grb2, hnRNP H, and e-FABP in carcinomas. Interestingly, Grb2 was not only overexpressed in breast carcinoma [18] and primary HNSCC tumours as shown here, but also in metastases thereof, making Grb2 an interesting tumour marker for detection and molecular targeting of MRD cells.

Heterogeneous ribonucleoprotein H belongs to a subgroup of pre-mRNA binding proteins [19]. These proteins are believed to play a fundamental role in the control of gene expression upon alternative splicing, as was shown for the kinase c-src [20], β-tropomyosin [21], and the thyroid hormone receptor [22]. Here, we demonstrate for the first time that hnRNP H is overexpressed in HNSCC and lymph node metastases. Of note, the expression of hnRNP H gradually increased with the neoplastic status, starting already in hyperplastic tissue. Thus, hnRNP H overexpression represents an early event during tumourigenesis. A possible contribution of the protein to tumour development remains to be elucidated. Interestingly, we identified hnRNP H as a 23 kDa protein in contrast to the expected theoretical molecular weight of 49.5 kDa. Similar findings have been reported by Honore et al. [23], who described the posttranslational cleavage of hnRNP H into a 22 kDa N-terminal and a 35 kDa C-terminal product. This is in line with our own results, as only N-terminal peptides were retrieved from mass spectrometric analysis of the 23 kDa protein spot corresponding to hnRNP H (data not shown).

E-FABP, also known as psoriasis-associated fatty acid-binding protein (PA-FABP) [24], is highly expressed in psoriatic epidermis [25], in different cancer entities [26,27], and was demonstrated to promote metastasis formation [28]. The analysis of e-FABP expression in healthy and diseased tissue of the upper aerodigestive tract confirmed a strong overexpression in HNSCC and metastases. Noteworthy, e-FABP was isolated with 3 out of 8 HNSCC sera, suggesting the presence of autoantibodies in a substantial amount of patients. Indeed, the screening of a larger panel of HNSCC sera resulted in a calculated sensitivity of 20.3% and a specificity of 97.9%, supporting the rationale of allo-AMIDA. Antibody titers were already detectable in patients with early stage disease (T1 and 2), possibly allowing a diagnostic measurement at early stages in combination with additional biomarkers.

In summary, we demonstrate that AMIDA can be applied both in an autologous or allogenic setting, yielding comparable results. Allo-AMIDA overcomes limitations of autologous screening techniques and broadens the spectrum of applications for the efficient detection of new tumour markers.

Acknowledgments

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