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Antibody response to a non-conserved C-terminal part of human histone deacetylase 3 in colon cancer patients

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Antibodies to cancer antigens can often be detected in the sera of patients, although the mechanism of the underlying humoral immune response is poorly understood. Using immunoscreening of tumor-derived cDNA expression libraries (SEREX), we identified human histone deacetylase 3 (HDAC3) as serologically defined antigen in colon cancer. Closely related HDAC1 and HDAC2 do not elicit humoral response in colon cancer patients. We show that the C-terminal region of HDAC3 protein lacking the homology to other Class I HDAC contains at least 3 distinct B-cell epitopes that are recognized by the serum antibodies. HDAC3 in combination with other SEREX antigens may become a useful molecular biomarker with diagnostic or prognostic value for a subset of colon cancer patients. (Supplementary material for this article can be found on the *International Journal of Cancer* website at http://www.interscience.wiley.com/jpages/0020-7136/suppmat/index.html).

Key words: antibodies; epitopes; HDAC3; serology

Human cancer antigens inducing T cell or B cell immunological responses are emerging molecular markers for disease diagnosis, monitoring and prognosis, as well as targets for active immunotherapy. Immunoscreening of tumor-derived cDNA expression libraries (SEREX) proved to be an efficient technique to clone serologically defined antigens.[1,2]

Histone deacetylases (HDAC) represent the family of enzymes involved in dynamic regulation of chromatin structure during transcription, but can also deacetylate a number of non-histone substrates.[3–9] A growing body of evidence implicates HDAC as playing an important role in carcinogenesis, particularly, in colorectal cancer, as suggested by expression profiling of colon cancer cells treated by HDAC inhibitor trichostatin A.[3–5] Histone deacetylases may be involved in carcinogenesis in different ways. Class I HDAC may downregulate tumor suppressors such as p53 and von Hippel-Lindau (VHL) gene product.[6–8] HDAC1 may also affect cancer progression by inhibiting estrogen receptor alpha (ER-α) protein expression and its transcriptional activity.[9] Recent experiments with siRNA for Class I and Class II HDAC demonstrated that the former (particularly HDAC1 and HDAC3) are implicated in regulation of proliferation and survival of cancer cells.[10] Overexpression of HDAC1 at mRNA and protein levels was reported for gastric and prostate tumors,[11,12] and aberrant expression of HDAC1 was detected in esophageal squamous cell carcinoma.[13]

We demonstrate that HDAC3, but not HDAC1 or HDAC2, is the target of humoral immune response in cancer patients. We cloned a novel antigen, MO-OVA-91 by serological screening of a cDNA library derived from the tumor of the ovarian cancer patient using autologous serum and identified it as a truncated form of histone deacetylase 3 (HDAC3), a Class I HDAC. Extensive serological analysis demonstrated the presence of anti-HDAC3 serum antibodies preferentially in colon cancer patients, as compared to ovarian cancer or other types of malignancies. Antibodies against HDAC3 were never observed in normal donors using SMARTA format of screening.[14]

We mapped corresponding B cell epitopes to a non-conserved C-terminal region of HDAC3 protein and carried out expression analysis to address the putative link between seroconversion and HDAC overexpression in tumors. In conclusion, HDAC3 seems to be a novel serologically defined biomarker that can be potentially used for diagnosis and for disease monitoring in colon cancer.

Material and methods

Specimens

Tissue samples from patients, undergone surgical resection of colorectal and ovarian cancer were provided by N.N. Blokhin Cancer Research Center (Moscow, Russia) and Municipal Hospital 24 (Moscow, Russia). Samples were either shock-frozen in liquid nitrogen and stored at −80°C or immediately stabilized in RNAlater tissue storage reagent (Ambion, Inc., Austin, TX) and stored at −20°C.

Normal sera obtained during routine diagnostic procedures were provided by out-patient clinic of Russian Ministry of Economics. Patients' sera collected before tumor resections were provided by N.N. Blokhin Cancer Research Center, Municipal Hospital 24 and Krankenhaus Nordwest (Frankfurt, Germany). Sera were aliquoted and stored at −80°C.

The study protocols were approved by the local Ethic Committees of participating clinical centers. The research activities at NCI-Frederick and at Moscow State University were designated exempt from Institutional Review Board (IRB) reviews.

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Sera pre-treatment for SEREX and SMARTA analysis

To remove antibodies reactive with *E. coli*- and phage-related antigens, sera (1:100 dilution in 0.2% non-fat dried milk (NFDM)/20 mM Tris-HCl, 150 mM NaCl (TBS) with 0.02% NaN_3) were pre-absorbed by overnight incubation with nitrocellulose membranes Hybond-C extra (Amersham, Little Chalfont, Buckinghamshire, UK) precoated with *E. coli* XL-1 Blue MRF' (Stratagene, La Jolla, CA)/control lambda ZAP phage (Stratagene) lysates. Pre-absorbed sera were stored at 4°C.

Sera pre-treatment for Western blotting

E. coli XLOLR bacteria were transformed with insertless pBK-CMV and grown in LB/Mg medium in the presence of 50 μg/ml kanamycin to A_{600} nm ~0.35 E. Protein expression was induced with 1 mM IPTG and cells were allowed to grow for an additional 4 hr at 37°C. Bacteria were spun down, resuspended in TBS with 0.2 mM protease inhibitor AEBSF-hydrochloride (Sigma, St. Louis, MO) (TBS/AEBSF) and disrupted by sonication. Bacterial lysate was diluted to a total protein concentration of 2 mg/ml in TBS/AEBSF. Tested sera were diluted in mixture of 2 parts of TBS with 5% non-fat dry milk (TBS/NFDM) and 1 part of bacterial lysate. Diluted sera were agitated 1 hr at room temperature (RT).

RNA extraction and construction of cDNA libraries

Total RNA was extracted from frozen ovarian tumor sample by TRI reagent (Sigma) and poly A^+ RNA was purified using the Oligotex mRNA Midi Kit (Qiagen, Valencia, CA). cDNA was obtained by ZAP Express cDNA synthesis kit (Stratagene), ligated into the lambda ZAP express vector and cloned using Gigapack III Gold Packaging Extract (Stratagene). Library containing 1–2 × 10^6 primary recombinants was amplified before immunoscreening.

Serological screening of tumor-derived cDNA expression library

Serological screening of tumor-derived cDNA expression library (SEREX) was carried out essentially as described earlier[1,15] with minor modifications. Briefly, a cDNA library was plated with *E. coli* XL-1 Blue MRF, host cells, transferred to the nitrocellulose membranes, incubated with autologous patient's serum (diluted 1/200) and alkaline phosphatase-conjugated goat anti-human Fcγ secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1/3,000. Reactive plaques were visualized with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

Serological mini-arrays of recombinant tumor antigens

Arrayed allogeneic screening was carried out by serological mini-arrays of recombinant tumor antigens (SMARTA) as described earlier.[14] Briefly, 0.5 μl drops of phage stocks (≥500 pfu/μl) were spotted directly to the LB agar plates containing a layer of solidified LB/0.8% agarose/5 mM IPTG premixed with *E. coli* XL-1 Blue MRF' cells. Plaque lysates were transferred to the nitrocellulose membranes and resulting arrays were incubated with allogeneic sera and developed as described above.

DNA sequencing

Phage cDNA clones were converted to pBK-CMV plasmids by *in vivo* excision procedure. Plasmid DNA was purified by alkaline lysis method and subjected to EcoRI/XhoI restriction enzyme digestion. Clones representing different cDNA inserts were sequenced using Thermo Sequenase II dye terminator cycle sequencing kit (Amersham) and ABI Prism automated DNA sequencer (Perkin Elmer, Wellesley, MA).

Bacterial expression of cloned proteins

E. coli XLOLR bacteria transfected by pBK-CMV plasmids were grown overnight at 37°C and intensive shaking (200*g*) in LB/Mg medium containing 1 mM IPTG and 50 μg/ml kanamycin. The cell lysates were tested by Western blotting.

Purification of recombinant MO-OVA-91

Plasmid pBK-CMV encoding MO-OVA-91 antigen was sequentially digested by Sal I and Spe I restrictases and ligated with His6 tag-encoding insert composed from the following primers: TCGACACATCACCATCACCATCACACA and CTAGTGTGTGATGGTGATGGTGATGTG. Transfected *E. coli* XL-1 Blue MRF' cells were grown in LB/Mg media in the presence of 50 μg/ml kanamycin to A600 nm ~0.35 E. Protein expression was induced with 1 mM IPTG and cells were allowed to grow for an additional 4 hr at 37°C, spun down and recombinant His6-tagged protein was batch-purified on Ni-NTA Agarose (Qiagen) in denaturing urea buffer according manufacturer's recommendations.

Western blotting

Total proteins were extracted from tissue samples by TRI reagent (Sigma). Proteins (10–30 μg per lane) were separated in 10% polyacrylamide gel in denaturing conditions, transferred to nitrocellulose membrane and incubated overnight with appropriate antibody or serum, washed 3–4 times and incubated for 1 hr with HRP-conjugated secondary antibodies (Jackson ImmunoResearch, diluted 1/20,000) in 1:2,000 dilution. Blots were developed using enhanced chemoluminescence (ECL) kit (Amersham). Anti-HDAC1 (rabbit, against peptide 466–482) and anti-HDAC2 (rabbit, against peptide 471–478) antibodies were purchased from Sigma, anti-HDAC3 antibodies were from Sigma (rabbit, against peptide 411–428) and BD Biosciences (San Jose, CA) (mouse, against peptide 309–425).

Northern blotting

Total RNA was extracted from tumor samples by TRI reagent (Sigma). RNA (20 μg per lane) was separated in 1% agarose formaldehyde gel, transferred to nitrocellulose membrane Hybond-N+ (Amersham) in 10 mM NaOH and hybridized with appropriate probes ^{32}P-labeled by random priming kit (Amersham) under stringent conditions (65°C) in Church and Gilbert buffer (131 mM Na_2HPO_4/119 mM NaH_2PO_4, pH 7.0, 7% SDS). After overnight hybridization membranes were sequentially washed at 65°C by Church and Gilbert buffer, 2× SSC, 2× SSC + 0.1% SDS, 0.5× SSC + 0.1% SDS and 0.2× SSC + 0.2% SDS. Blots were analyzed by Cyclone™ Storage Phosphor System (Packard Instruments, Meriden, CT). Class I HDAC cDNA 3′-fragments for ^{32}P-labeling were obtained by RT-PCR using total ovarian RNA as template, oligo$(dT)_{15}$ primer for first chain synthesis and following primers for PCR: HDAC1 forward AAGAAAGAAGTCACCGAAG, reverse CTAGACTAGCAACCTCCAC; HDAC2 forward CGAAGAAATGTGGCTGATC, reverse GCTCAGAAAGGCCAATTAC; HDAC3 forward CAATGACAAGGAAAGCGAT, reverse CCTTGTCTACCCGTTCATC.

Cloning of cDNA for Class I HDAC

For cloning of full length HDAC1, HDAC2 and HDAC3 the appropriate cDNAs were amplified by RT-PCR using total ovarian RNA as template, oligo$(dT)_{15}$ primer for first chain synthesis and following primers for PCR: HDAC1 (aa5-482) forward: GGC**GAATTC**GCAGGGCACCCGGAGGAAA, reverse: CGT**CTCGAG**CTCAGCAGGAAGCCAGAGC; HDAC2 (aa2–488) forward: GGA**CAATTG**GGCGTACAGTCAAGGAGGC, reverse: AAA**CTCGAG**CACATAAAGCATGGTGGA; HDAC3 (aa4–428) forward: CAT**GAATTC**GACCGTGGCCTATTTCTACGA, reverse: ATT**CTCGAG**GACACAGCATCCCAAGCCAC.

PCR products were subjected to restriction by EcoR I/XhoI for HDAC1 and HDAC3 or by MunI/XhoI for HDAC2, ligated into the lambda ZAP express vector and packed to Gigapack III Gold Packaging Extract (Stratagene).

Identification of B-cell epitope-containing region of HDAC3

The cDNA fragment encoding HDAC3 (aa4–428) was digested with 3.3 ng/ml of DNase I (Sigma) in presence of 10 mM $MnCl_2$ for 20 min at 22°C. Reaction was terminated

TABLE I – SERUM REACTIVITY OF THE SEREX-DERIVED CLONES MO-OVA-91 AND NY-CO-9 AND CLONED CLASS I HDAC WITH SERA FROM NORMAL DONORS AND CANCER PATIENTS[1]

Clone	Sera						
	Normal donors	Colon cancer	Ovarian cancer	Breast cancer	Renal cancer	Head and neck cancer	Melanoma
MO-OVA-91	0/100	10/194[2]	2/90	0/60	1/51	0/31	0/45
HDAC1	0/90	0/90	0/75	0/12	0/51	0/31	0/45
HDAC2	0/90	0/90	0/75	0/12	0/51	0/31	0/45
HDAC3	0/90	10/194[3]	2/75	0/12	1/51	0/31	0/45
NY-CO-9 (HDAC5)	0/100	0/194	0/75	0/60	0/51	0/31	0/45

[1]The sera that reacted with MO-OVA-91 also reacted with HDAC3. MO-OVA-91 positive sera from ovarian cancer patients include autologous serum used for screening of tumor-derived library. None of tested sera reacted with NY-CO-9, a SEREX clone encoding class II HDAC5 (kindly provided by Dr. M.J. Scanlan), a colon cancer antigen discovered in the earlier study.[15,16] –[2] $p < 0.02$. –[3] $p < 0.05$.

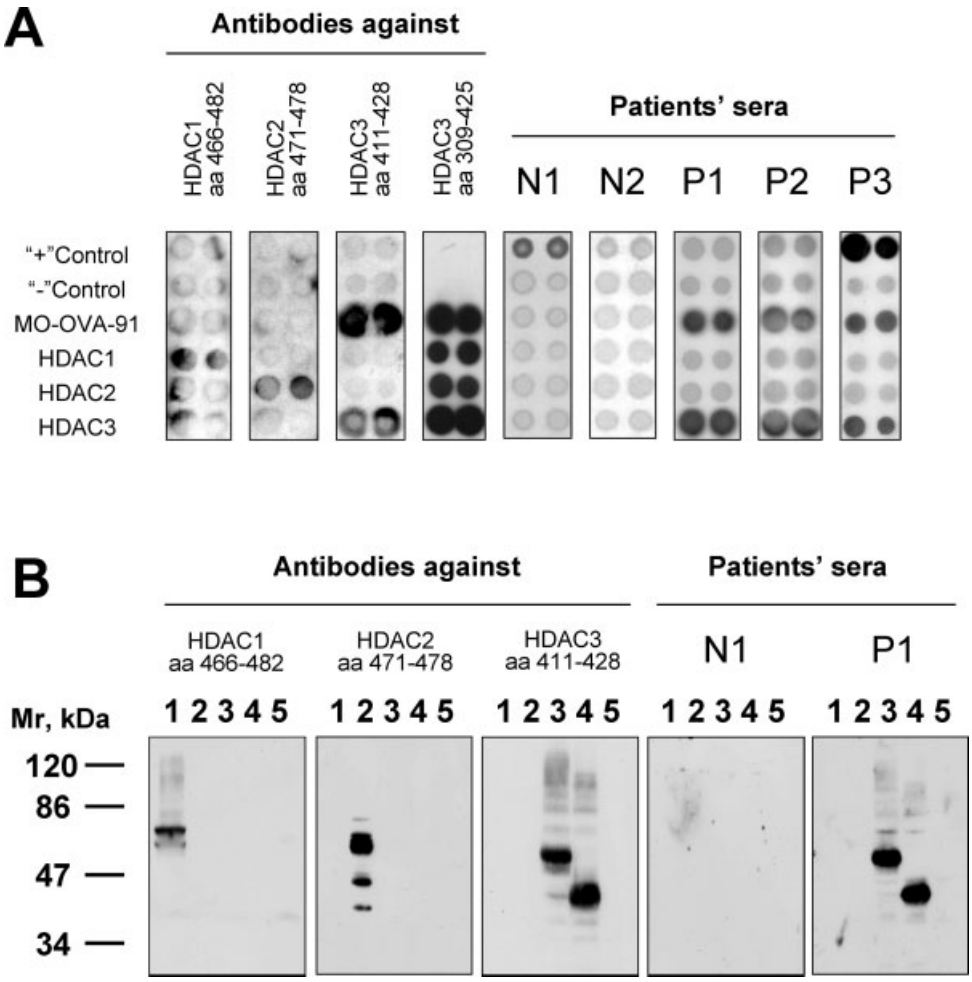

FIGURE 1 – Serological reactivity of Class I HDAC. (*a*) Cross-reactivity and specificity assayed by SMARTA. (+) Control, clone encoding SWAP-70 (GenBank accession number AF210818). Antibodies to SWAP-70 frequently (about 60%) occurs in human sera. (−) Control, empty vector; N1 and N2, sera of MO-OVA-91 serologically negative patients; P1–P3, sera of MO-OVA-91 serologically positive patients. All antibodies diluted 1/1,000, all sera diluted 1/200. (*b*) Western analysis of recombinant Class I HDAC expressed in XLOLR *E. coli*. 1, HDAC1; 2, HDAC2; 3, HDAC3; 4, MO-OVA-91; 5, empty vector; N1, serum of MO-OVA-91 serologically negative patient; P1, serum of MO-OVA-91 serologically positive patient. All antibodies diluted 1/1,000, all sera diluted 1/500.

by adding of EDTA to the final concentration 5 mM. Digested DNA was size-fractioned by electrophoresis and 150–400 bp fragments were blunt-ended by T4 DNA polymerase (Sibenzyme, Russia), phosphorylated by T4 DNA kinase (Sibenzyme) and ligated to synthetic EcoRI and XhoI adapters by T4 DNA ligase (Sibenzyme). The adapters were obtained by annealing the following pairs of primers: GGCAAGG-**GAATTC**TTTGGAG and CTCCAAA**GAATTC**CCTTGCC for EcoRI adapter and GCGTACT**CTCGAG**ACGTGCT and AGCACGT**CTCGAG**AGTACGC for XhoI adapter. The fragments with ligated adapters were subjected to EcoRI/XhoI digestion, ligated into lambda ZAP express vector and packed using Gigapack III Gold Packaging Extract (Stratagene). To map the B cell epitopes, the sub-library containing 1–2 × 10^4 primary recombinants was amplified and screened by sera of MO-OVA-91 (HDAC3)-positive patients as described above.

Peptide scanning analysis of C-terminal part of HDAC3

A custom made set of 15-mer peptides (offset by 3 amino acids, 13 peptides) spanning the last 50 amino acids of HDAC3 C-terminus was purchased from Mimotopes Pty. Ltd. (Clayton, Victoria, Australia) (Fig. 2). A spacer sequence (SGSG) and a biotin residue were added to the N-terminus of each peptide. Plates were coated with 5 μg/ml of streptavidin diluted in water, 100 μl per well or 3 μg/ml of truncated recombinant HDAC3 (corresponding to the part encoded by MO-OVA-91 SEREX clone) diluted in water, 100 μl per well. Plates were left under laminar flow to dry and were either used immediately or stored at 4°C in a

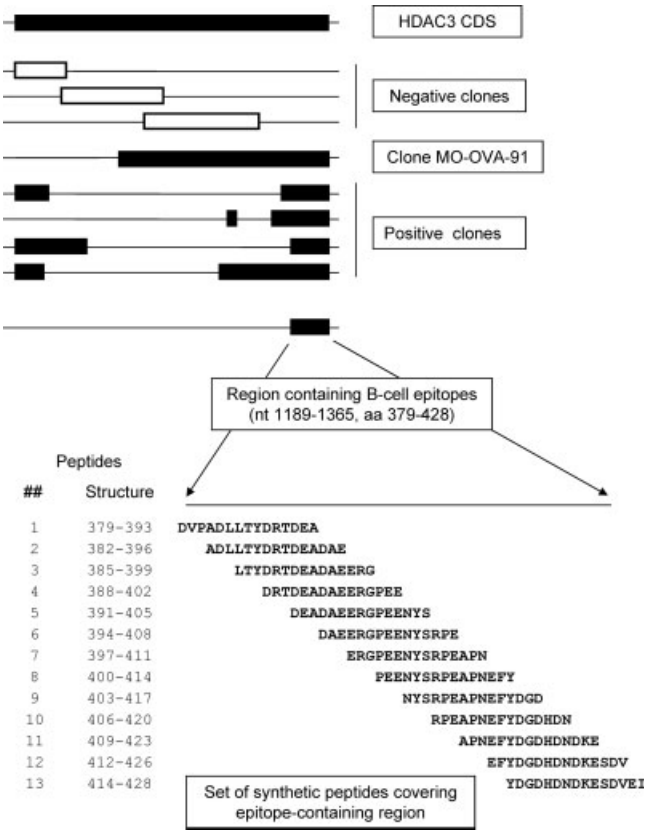

FIGURE 2 – Mapping of B-cell epitopes of HDAC3 recognized by cancer sera. Scheme of deletion mutants isolated from HDAC3 sub-library and peptides covering candidate epitope-containing region.

sealed bag. Plates were washed 4 times in PBS containing 0.1% (v/v) Tween 20 (PBST) and subsequently blocked with PBS containing 2% BSA for 1 hr at room temperature. Plates were then washed 4 times in PBST, and streptavidin-coated wells were incubated with 100 μl of each biotinylated peptide (5 ng/μl) in PBS, containing 0.1% BSA and 0.1% NaN_3 (PBS/BSA/NaN_3) for 1 hr at room temperature and washed 4 times with PBST. Plates were incubated overnight at 4°C with sera diluted by PBS/BSA/NaN_3 (dilutions from 1/100 to 1/1,000 as indicated), washed 4 times and incubated 1 hr at room temperature with AP-conjugated secondary antibodies (Jackson ImmunoResearch) in 1:5,000 dilution in PBS containing 2% BSA. Plates were then washed 4 times with PBST and developed with ''Sigma Fast'' *p*-nitrophenol phosphate soluble substrate (Sigma).

For inhibition assay diluted tested sera were pre-incubated with streptavidin (final concentration 30 μg/ml) and appropriate peptide (final concentration 5 μg/ml) for 1 hr at room temperature and analyzed by ELISA as described.

Results

Initial identification of SEREX clone MO-OVA-91 as a fragment of human HDAC3

We used SEREX methodology to identify and characterize novel candidate tumor antigens expressed in several types of human cancers.[14,17–20] In particular, one of the positive clones from an ovarian cancer-derived cDNA library, screened with autologous serum, MO-OVA-91, was identified by sequence analysis as a partial transcript of human *HDAC3* gene lacking 448 bp of the complete cDNA at the 5′-region. The deduced amino acid sequence of MO-OVA-91 corresponded to HDAC3 amino acid residues 132–428. No mutations in the coding region were detected in the clone MO-OVA-91 as compared to human *HDAC3* gene sequence (Reference sequence NM_003883).

HDAC3 is a serologically defined colon cancer antigen

To determine the frequency and specificity of immune response against HDAC3, sera from normal individuals and from patients with ovarian, colon, renal, head and neck, breast cancers and with melanoma were tested for their seroreactivity against clone MO-OVA-91 (Table I). Interestingly, a low incidence of serological response in the SEREX format (10/194, or 5%) to MO-OVA-91 (HDAC3) was found predominantly in sera from colon cancer patients. The patient who was the source of tumor sample was not available to evaluate the possibility of other malignancies. Only one additional MO-OVA-91 positive serum was found among 90 ovarian cancer sera tested, and a single positive serum was also found in the collection of 51 sera from patients with renal cancer. Importantly, antibodies to MO-OVA-91 (HDAC3) were not detected in any of 100 normal donors sera. Therefore, after serological analysis MO-OVA-91 (HDAC3) emerged as a colon cancer-related antigen.

Sera from MO-OVA-91-positive patients specifically recognize HDAC3 but neither HDAC1 or HDAC2

Commercially available mouse anti-HDAC3 antibodies against peptide containing amino acids 309–425 of human HDAC3 recognized not only the target protein but also 2 additional bands of higher molecular weights (presumably, HDAC1 and HDAC2; Supplementary Fig. 1*a*) (Supplementary material for this article can be found on the *International Journal of Cancer* website at http://www.interscience.wiley.com/jpages/0020-7136/suppmat/index.html). We addressed a possible serological cross-reactivity amongst several Class I HDAC that may be important for the interpretation of MO-OVA-91 reactivity.

Expression clones containing virtually full-length cDNAs for HDAC1 (aa5–482), HDAC2 (aa2–488) and HDAC3 (aa4–428) were prepared in lambda ZAP vector. pBK-CMV-based recombinant phagemids were excised *in vivo* and used for transfecting XLOLR *E. coli* cells. The results of subsequent serological analysis of the clones encoding 3 Class I HDAC using SMARTA technique[14] and by Western blotting are shown in Figure 1*a*,*b*, respectively. Analysis of sera from 10 MO-OVA-91 (HDAC3)-positive patients demonstrated that they recognized only HDAC3, not the other tested HDAC (Table I). At the same time, mouse anti-HDAC3 antibodies raised against peptide 309-425 clearly reacted with HDAC1 and HDAC2 as well (Fig. 1*a*). We next evaluated the serological reactivity of HDAC1 and HDAC2 expression clones and detected none in panels of sera from either cancer patients (including colon cancer patients) or normal donors (Table I). Finally, we verified that the pattern of serological reactivity of the full-length HDAC3 clone against our sera panel was identical to that of the original MO-OVA-91 clone (Fig. 1*a*, Table I). HDAC3 emerged from this analysis as the only representative of Class I HDAC with a cancer-related serological profile.

Mapping of HDAC3 B-cell epitopes

To understand specificity of serological response to HDAC3, as compared to other Class I HDAC, we decided to map HDAC3 B-cell epitopes recognized by sera of colon cancer patients. For preliminary identification of candidate epitope-containing regions we generated HDAC3 expression sub-library by digestion of whole length HDAC3 cDNA by DNase I, size-fractioning and cloning of 150–400 bp fragments into lambda ZAP express vector. The resulting sub-library was screened by pooled sera from 3 patients positive for MO-OVA-91. Analysis of positive clones (Fig. 2) allowed us to roughly map the B-cell epitopes recognized by patient antibodies to the C-terminal part of the HDAC3 (amino acids 379–428). Importantly, all

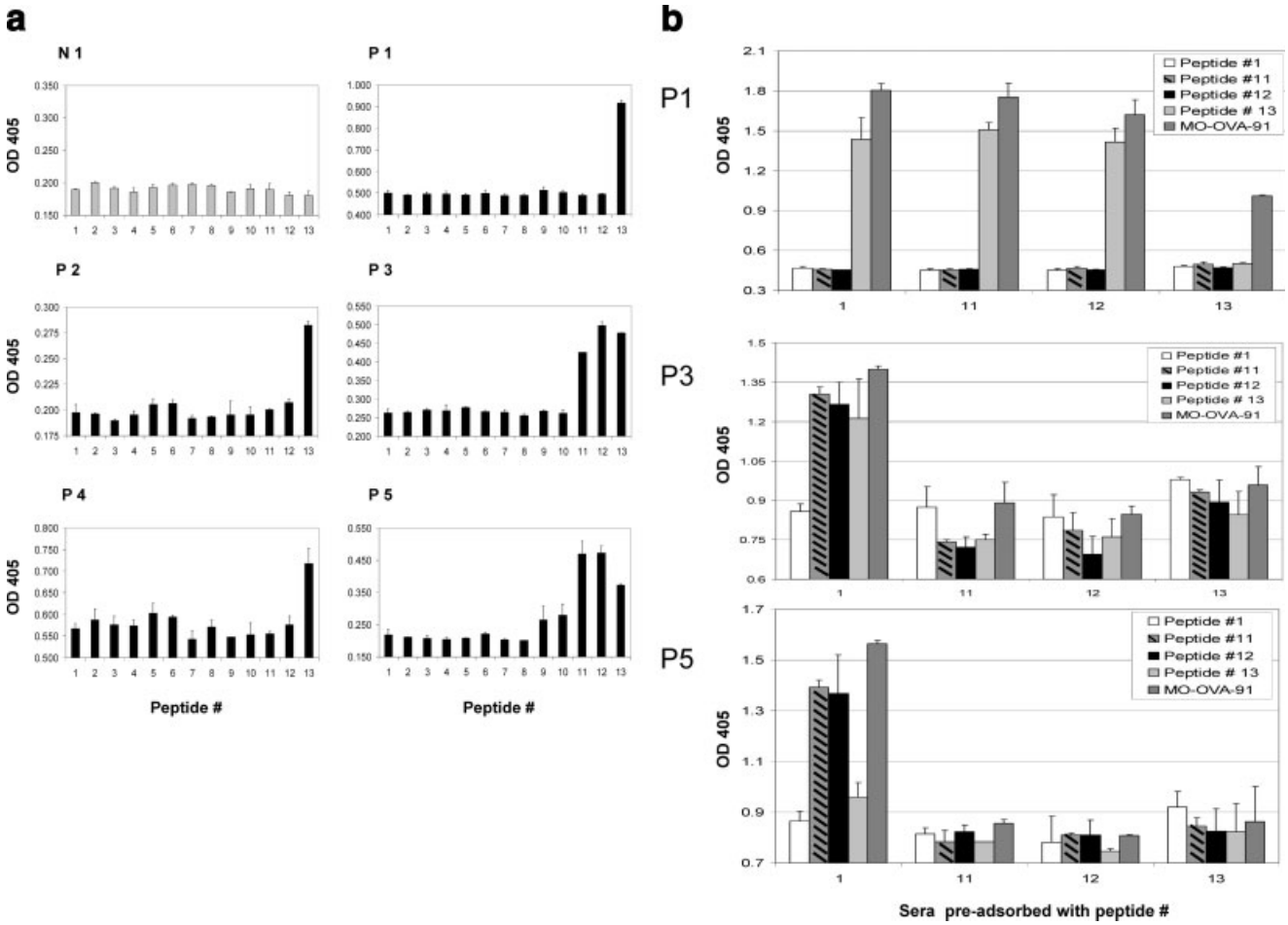

FIGURE 3 – Peptide scanning analysis of the C-terminal part of HDAC3. (*a*) Representative ELISA of MO-OVA-91 negative (N1) and positive (P1-P5) sera form patients with colon cancer. All sera were diluted 1/300 except P1 (1/500) and P4 (1/100). (*b*) Reactivity of MO-OVA-91 positive sera P1 (1/1,000), P3 and P5 (both 1/300) after exhaustion of peptide-specific antibodies.

MO-OVA-91 positive sera tested recognized the same region of HDAC3 molecule.

For more precise mapping of HDAC3 B-cell epitopes we carried out ELISA analysis of short peptides covering epitope-containing region identified previously. We screened the set of biotinylated 15-mer peptides (with offset by 3 amino acids) covering amino acids 379–428 with sera from MO-OVA-91 positive and negative patients (Fig. 2). Results of ELISA analysis demonstrated that at least 2 epitopes localized at the very C-terminus of HDAC3: the first one within the amino acids 412–423 (EFYDGDHDNDKE, the overlapping part of peptides 11 and 12) and the second one at the very C-terminus including the 2 last aminoacids 427–428 (inside peptide 13) (Fig. 3). The region containing peptides 11–13 (amino acids 412–428) is not conserved between HDAC3 and HDAC1/HDAC2 (Fig. 4), providing possible explanation for the distinct serological reactivity of HDAC3 as compared to other Class I HDAC.

To investigate the relationship between the C-terminal epitopes, we carried out inhibition analysis by prior removal of antibodies recognizing specific peptides from MO-OVA-91 positive sera. As shown in Figure 3*b*, the exhaustion of serum P1 (reacting only with peptide 13) against peptides 11 and 12 had no effect on reactivity to peptide 13. In contrast, exhaustion of serum P3 (reacting with peptides 11–13) against peptides 11 and 12 abolished reactivity to peptide 13 and *vice versa*. Surprisingly, reactivity of serum P5 (reacting only with peptides 11 and 12) was inhibited by either of peptides 11–13. These results indicate that C-terminus of HDAC3 contains at least 3 distinct epitopes that can be recognized by sera of colon cancer patients.

Overexpression of Class I HDAC in colon cancer tissues does not correlate with seroconversion

To address the possibility that HDAC3 and perhaps other Class I HDAC may be overexpressed in tumors from colon cancer patients, positive for MO-OVA-91 antigen, we evaluated protein and mRNA levels in tumor and control samples. Protein levels for HDAC1, HDAC2 and HDAC3 in colon tissues were determined by Western blotting of tumor and adjacent non-cancerous control tissue samples. Representative results of Western blotting are shown in Supplementary Figure 1. When compared to normal colon mucosa, detectable (2-fold and higher) increases in the protein expression levels of HDAC3 in tumor tissues were observed in 3 of 8 (38%) of MO-OVA-91 serologically positive patients and in 13 of 40 (33%) of MO-OVA-91 serologically negative patients. Interestingly, the overexpression of HDAC1 and HDAC2 was observed in somewhat higher portion of patients, regardless of their serological reactivity to MO-OVA-91 (Supplementary Table I). We also examined the levels of Class I HDAC mRNA expression by Northern blot analysis (representative results are shown in Supplementary Fig. 2) and observed no significant differences between matched normal and tumor tissues regardless of the serological reactivity to MO-OVA-91. We concluded that the

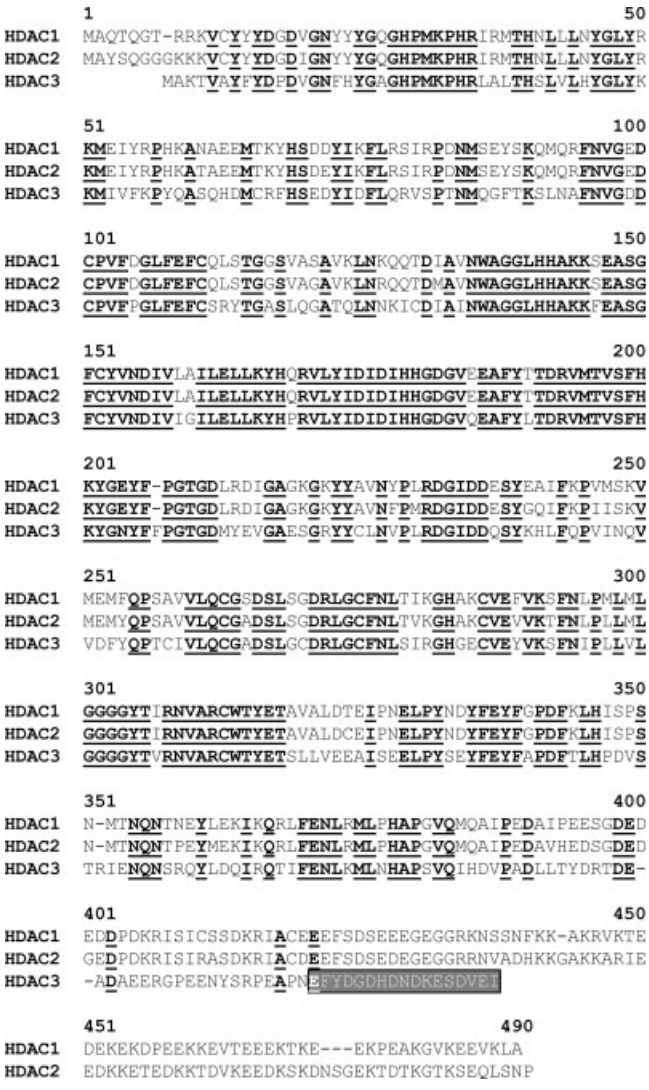

FIGURE 4 – HDAC1–3 amino acid sequence alignments and the position of HDAC3 B-cell epitopes. Region 412–428 in HDAC3 (gray background) lacks the homology (bold underlined) to HDAC1 and HDAC2.

modest increase in protein levels in tumors was probably due to posttranscriptional regulation of Class I HDAC. The specificity of the reaction toward HDAC3, as opposite to HDAC1 and HDAC2, cannot be explained by the pattern of the overexpression.

Discussion

The growing interest to histone deacetylases as molecular targets for cancer therapy is based on numerous studies implicating the role of HDAC in carcinogenesis.[3,4] Initially, the role of HDAC in colon cancer development was suggested due to the fact that high-fiber diets are associated with a significant decrease in the incidence of colon cancer, whereas butyrate, a 4-carbon fatty acid, produced in millimolar concentration by the bacterial fermentation of fiber is a potent inhibitor of histone deacetylases.[3,21] Several other inhibitors of HDAC, such as MS-275, TSA and FR901228, are being evaluated clinically.[22] Other inhibitors, such as Scriptaid, NVP-LAQ824 and others, were recently proposed for clinical use for colon cancer and other malignancies, including lymphomas.[23,24] Confirmation of HDAC role in gene regulation in human colorectal cancer was provided recently by a study in which inhibition of histone deacetylases (with or without demethylation of DNA) led to increased expression of substantial number of genes that are epigenetically silenced in this type of malignancy.[5]

Despite significant similarities to other Class I HDAC (especially to HDAC1 and HDAC2), HDAC3 possesses several unique features. In particular, HDAC3 can be found in the nucleus and in the cytoplasm, whereas all other Class I HDAC are strictly nuclear proteins.[25,26] Additionally, unlike HDAC1 and HDAC2, the disruption of HDAC3 gene, which is homologous to yeast protein RPD3,[27] makes DT40 chicken cells non-viable,[25] suggesting specific and non-redundant function of this gene product. Another interesting feature of the human HDAC3 was reported recently by Zhang *et al*.,[28] who demonstrated that HDAC3 is necessary and sufficient for silencing the growth-differentiation factor 11 (Gdf11) gene, a TGF-β family member that inhibits cell proliferation.

We demonstrated that sera of some colon cancer patients recognize only HDAC3 and not HDAC1/HDAC2, and we mapped the corresponding B cell epitopes to a non-conserved C-terminal part of HDAC3. Mutations may be a cause of the antibody response, but no sequence variations in HDAC3 in the available tumor samples were detected so far. Interestingly, in another study HDAC5, a representative of Class II HDAC, was identified as a colon cancer antigen, NY-CO-9.[15,16] None of the 194 colon cancer sera used in our study was positive for NY-CO-9 using recombinant phage assay in SMARTA format.[14]

Not only HDAC3, but all 3 best-studied Class I HDAC are overexpressed frequently in tumors of patients with colorectal cancer, in agreement with the earlier reports on the HDAC1 overexpression in gastric and prostate cancers.[11,12] In colon cancer we found that the protein levels of HDAC1, HDAC2 and HDAC3, but not their mRNA levels, were increased in tumors as compared to non-cancerous colon mucosa from the same patients. Of note, overexpression of HDAC1 and HDAC2 in colorectal tumors was reported recently by Huang *et al*.,[29] who used alternative techniques to estimate mRNA and protein levels (quantitative real-time RT-PCR and immunohistochemistry, respectively).

We were unable to establish a statistically significant correlation between seroconversion and HDAC3 levels in tissues, but this may be due to the current sensitivity of serological assays. The sensitivity will likely be increased in the future due to the use of recombinant proteins, rather than recombinant phage lysates. In this regard, it should be pointed out that 5% for HDAC3 reactivity in colon cancer is the conservative minimal estimate based on the sensitivity of the SEREX/SMARTA format serology. Combination of dozens of rare cancer antigens on the array may result in development of a useful diagnostic tool.

In conclusion, our study has identified HDAC3 as a novel serological antigen/biomarker for colon cancer.

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