Generation and characterization of monoclonal antibodies against choline kinase α and their potential use as diagnostic tools in cancer

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Abstract. Choline kinase α (ChoKα) is a metabolic enzyme involved in the synthesis of phosphatidylcholine, recently implicated in cancer onset since it is overexpressed in a variety of human cancers such as mammary, lung, colorectal and prostate adenocarcinomas. Furthermore, overexpression of ChoKα in human HEK293T cells confers them oncogenic properties with the induction of tumors after subcutaneous injection in nude mice. ChoKα levels in tumor samples have been analyzed using polyclonal antibodies and Western blotting. These techniques have considerable limitations and do not allow for a precise and efficient evaluation of the real significance of ChoK overexpression in human carcinogenesis. We developed a set of monoclonal antibodies with high specificity and sensitivity against ChoKα, and characterized their properties. We provide evidence that the newly generated MoAbs against ChoKα have potential use in cancer diagnosis by conventional immunohistochemistry techniques.

Introduction

Choline kinase α (ChoKα), is the first enzyme in the Kennedy pathway for the biosynthesis of phosphatidylcholine (PC), the major phospholipid of biological membranes. There is strong evidence demonstrating the implication of ChoK and its related product, phosphorylcholine (PCho), in the process of carcinogenesis (1-4).

ChoK is up-regulated in a high percentage of human tumor-derived cell lines as well as in different human tumoral tissues such as breast, lung, colon and prostate (5,6). Taken together, these tumors represent >70% of the total number of cancer cases in developed countries. The incidence of overexpression or increased activity of ChoK in these four types of cancer ranges from 40% to 60% (5-7), suggesting that ChoKα may represent an important novel tumor marker, useful for either diagnosis, prognosis or response to treatment.

Early and reliable procedures for cancer diagnosis provides valuable information that can improve mangement of this disease, helping to achieve an increase in life expectancy. Therefore, the development of reagents and appropriate methodologies that allows the determination of ChoK over-expression in human tumors, could represent a significant advance in the diagnosis of cancer. Moreover, it has been demonstrated that inhibition of ChoKα represents an effective antitumoral strategy with a novel mechanism of action (8-13), and that the involvement of ChoK in carcinogenesis may be linked to an increase in its expression in tumor cells (4-7). Thus, an available methodology that could be used for the determination of ChoK levels in human tissue samples could be also useful for the identification of potential candidates for therapy using ChoK inhibitors. Monoclonal antibodies (MoAbs) are extensively used in biomedical research and diagnosis of many diseases including cancer, and provide high sensitivity and specificity, both needed for such studies.

We developed a set of monoclonal antibodies against ChoKα useful as tools in several molecular biology techniques as well as in immunohistochemical studies. Finally, we provide preliminary evidence for the potential use of these MoAbs as diagnostic tools in cancer.

Materials and methods

Antigen preparation and purification. Human ChoKα expression and purification was performed as described previously as a fused GST-ChoKα (5,14,15). A standard procedure for purification by glutation sepharose method was followed according to the manufacturer's instructions (Amersham Biosciences Europe, Germany). Partially purified ChoKα was resolved in an SDS-PAGE. The gel was sliced

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off and the piece containing ChoK homogenized in PBS with complete Freunds Adjuvant (Sigma-Aldrich, MO) (1:1; vol:vol).

Balb/c mice immunization. Five 8-week-old mice were inoculated intraperitoneally with 0.5 ml of the antigen homogenate containing 100 μg of recombinant purified ChoKα. Two weeks later, the first boost was inoculated in the same manner with 100 μg of recombinant purified ChoKα but in this case mixed with Incomplete Freund Adjuvant (Sigma-Aldrich). This process was carried out 2 more times.

Cell fusion. Mice with a positive immune response towards ChoKα were identified by Western blotting against the partially purified protein. Selected mice were prepared to spleenocyte fusion 3 days after the last booster. Spleens were aseptically removed from each mouse and poured into a dish containing 10 ml of Dulbecco's modified Eagle's medium (DMEM) without serum, the tissue was teased until most of the cells were released, and then the spleenocytes were collected into a tube discarding larger pieces of tissue (16). A total of 10^8 spleenocytes were used for each fusion with 10^7 NS-1 myeloma cells. After 24-h incubation, cells were splited into fresh medium supplemented with 10% FBS at a final concentration of $5x10^5$ cells/ml. For cell fusion we used 1 ml of DMEM without serum containing 0.5 g of PEG 1500 (Roche) at 50˚C.

Selection and cloning of hybrid colonies. Successful hybrids were selected in HAT supplemented medium as recommended by the manufacturer (Gibco, Invitrogen, CA). Hybridoma colonies were cloned by two consecutive rounds of the standard minimal dilution technique.

ELISA. Multi-well microtitration plates (Nunc, Germany) were coated using 50 μl/well of 5 μg/ml recombinant ChoKα or GST in distilled water. After 1 h of incubation at 37˚C, plates were washed with PBS/0.05% Tween-20, and blocked with 10% milk in PBS overnight at 4˚C. Plates were washed and incubated with 50 μl/well of the supernatant for 1 h at 37˚C. Wells were washed, and a goat anti-mouse HRP (horseradish peroxidase) secondary antibody (Dako Cytomation, CA) added at 1:1000 dilution. Plates were incubated for 1 h at 37˚C, washed and developed using the colorimetric substrate ABTS solution (Bio-Rad, CA). The reaction was stopped by addition of 100 μl of SDS 0.5%.

Cytospin. A total number of $2.5x10^4$ transfected HEK293T cells were plated on a slide and loaded in a cytospin cuvette, and centrifuged at 800 rpm for 3 min. An immunodetection assay was carried out in adhered cells. After blocking non-specific binding sites with a peroxidase blocking solution, slides containing the adhered cells were incubated with the supernatants from hybrid colonies for 30 min at room temperature. After two washes with PBS a secondary anti-mouse antibody was applied for 1 h at room temperature and developed by standard procedures using DAB.

Cell cultures. Human embryonary kidney cells, HEK293T, were used as a model to monitor the selection of hybrid colonies and hybridomes and for testing the production of anti-ChoKα antibodies. NS-1 myeloma cell line was used for cell fusion. Mouse fibroblast NIH3T3, and NIH3T3-Tmar that stably overexpress murine ChoK were used for testing the cross-reactivity with murine choline kinase. Cell cultures were maintained in 10% serum supplemented media at standard conditions of temperature and humidity, at 5% of CO_2.

Tissues and cell lysis. Tumor tissues were extracted from surgical biopsies of patients previously diagnosed with cancer from Hospital La Paz (Madrid, Spain). Samples were homogenized and lysed in buffer containing 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3 M NaCl, 25 mM HEPES pH 7.5, 20 mM ß-glycerophosphate and 0.1% Triton X-100 with a cocktail of protease inhibitors.

Western blot analysis. Equal amounts of cell lysates (30 μg) were resolved by electrophoresis in a 10% SDS-PAGE as previously described (5). The specific binding was detected using a chemiluminescence detection kit ECL (Amersham) following the manufacturer's instructions.

Enzymatic activity assay. Choline kinase assays were performed as described (12,14) in the presence of methyl [^{14}C]-choline chloride (50-60 μCi/mmol, Amersham Biosciences Europe) at 37˚C for 30 min.

Immunoprecipitation. Hybridome culture medium was diluted 1:100 in lysis buffer and incubated with previously BSA-blocked Protein-G coated microbeads (Sigma-Aldrich) for 1 h at 4˚C. After three washes in lysis buffer, cell lysates were immunoprecipitated with the MoAbs linked microbeads. To quantify the relative capability of the MoAbs produced, we performed an *in vitro* ChoK activity assay in the immunoprecipitates as described (12,14).

Immunohistochemistry. Sections of 5 μm were cut from formalin-fixed, paraffin-embedded tissue blocks. Slides were deparaffinated and endogenous peroxidase activity blocked by incubation in 3% H_2O_2 in methanol for 10 min at room temperature. Antigens were retrived by incubation in 940 μM EDTA, pH 7.2, for 45 min at 155˚C. The primary anti-ChoK monoclonal antibody was diluted at 1:2 in 1% BSA TBS. Tissue slides were incubated for 1 h at room temperature. Slides were rinsed in TBS and incubated with the peroxidase based EnVision™ kit (Dako Cytomation) for 30 min at room temperature. Specimens were then incubated with diaminobenzidine chromogenic substrate (Dako Cytomation) for 5 min at room temperature. Sections were counterstained with hematoxylin. About 10 million HEK293T cells overexpresing human ChoKα were centrifuged and paraffin embedded following standard procedures. After 24 h of FAA fixation, cell pellets were dehydrated starting with 50% ethanol for 30 min and gradually increasing alcohol concentration to 100%. Pellets were placed into xylene after embedding in liquid paraffin.

Results

Mice were inoculated with purified ChoKα and positive sera were identified by Western blots (data not shown). Spleens

Table I. Summary of the main features of anti-ChoKα mAbs generated.

MoAb	WB	IP	Molecular activity post-IP	IHC	Iso-type	Mouse cross-reactivity
AD1	++	++	+++	+++	IgG1.κ	No
AD2	++	++	++	+++	IgG1.κ	No
AD3	+++	++	-	+++	IgG1.κ	No
AD4	+++	++	+++	++	IgG1.κ	No
AD5	+++	++	+++	+++	IgG1.κ	No
AD6	+++	-	-	+	IgG1.κ	Yes
AD7	+++	++	+++	+	IgG1.κ	No
AD8	+++	++	-	+	IgG1.κ	No
AD9	+++	-	-	-	IgG1.κ	Yes
AD10	+++	-	-	+	IgG1.κ	No
AD11	+++	+	-	-	IgG1.κ	Yes
AD12	+++	-	-	-	IgG1.κ	No
AD13	+++	++	+++	-	IgG1.κ	No
AD14	-	++	++	++	IgG2a.κ	No

An arbitrary +/- quantification system was applied to Western blot (WB), immunoprecipitation (IP) and immunohistochemistry (IHC) analysis. +++ indicates excellent, ++ good, + low, and - no sensitivity for this technique. Enzymatic activity of ChoK in the immunoprecipitates and murine ChoK cross-reactivity are also indicated.

were removed from positive mice and fused to myeloma cells. The third week after cell fusion, 149 hybrid colonies were screened for antibody production. Due to its simplicity and the high number of samples that we could check at the same time, selection of positive immunoglobulin producing hybrid colonies was carried out by ELISA. Most of the culture supernatants of hybrid colonies presented positive reaction against ChoKα antigen, but only colonies that showed stronger colour reaction than the polyclonal antibody were selected for further analysis. Thus, 19 hybridome colonies were selected and cloned using the minimal dilution technique. To ensure a monoclonal culture, two consecutive rounds of subcloning in a 96-well plate was performed. A total of 49 positive clones were obtained and further tested for antibody production.

Monoclonal antibody production screening. Selection of positive immunoglobulin producing clones was carried out by two consecutive methods: ELISA and cytospin immunostaining. ELISA was carried out to confirm the results obtained during the cloning process. From the initial 49 clones checked for anti-ChoKα antibody production, we selected 14 clones that strongly recognized the antigen by ELISA (data not shown).

To further confirm these results and to ensure that antibodies selected could recognize ChoKα in native conditions, we carried out a cytospin immunostaining (data not shown). Human embryonary kidney 293T cells (HEK293T) transiently transfected with the ChoKα gene-encoding vector were used for screening, using as a negative control cells transfected by pCDNA3b empty vector. In this case, transfected cells were adhered to a slide by centrifugation and an immunodectection (immunostaining) assay using the hybridome culture medium as a primary antibody was carried out. The results of the cytospins confirmed that all the 14 hybridomes selected produce antibodies that effectively recognize ChoKα under native conditions. A total of 14 different clones scoring positive for the production of anti-ChoKα immunoglobulin by these two screening methods were selected and subsequently characterized. The results of the characterization are summarized in Table I.

Isotype determination. Determination of the class and subclass of antibody is useful in predicting the properties of the antibody, and are needed for their proper use. Knowledge of those properties facilitates choosing correctly the secondary antibody that recognizes the MoAbs produced, or the kind of resin that effectively binds the antibody. Furthermore, it is also a first indication of monoclonality (17).

According to the immunization schedule, we expected a secondary immune response in treated mice, where antibodies produced are predominantly of the Immunoglobulin G class (18). IgG antibodies often show higher affinities for the antigen that other classes generated in primary response. Table I shows that heavy-chains of the MoAbs generated are γ-chains subclass 1 except AD14 that is subclass 2a, all forming immunoglobulin-G class antibodies. Light-chains were all κ class. Therefore anti-mouse IgG was used as a secondary antibody for further experiments.

Western blot analysis. Immunoblots are essential to truly establish the specificity of the antibodies for the protein of interest (17,18). We investigated the potential use of the MoAbs produced in WB. To that end, HEK293T cells were transfected with the plasmid encoding for the human ChoKα gene or with an empty vector as negative control. Equal amounts of cell lysates were resolved in an SDS-PAGE and transferred to a nitrocellulose membrane. Each hybridome selected culture medium was incubated separately, and as a positive control a polyclonal anti-serum generated against human ChoKα, described previously, was used (1). Thirteen out of the 14 selected media scored as positive clones. A representative experiment is shown in Fig. 1A. No cross-reacting or non-specific bands were observed, an indication of their specificity.

Additionally, we tested whether the generated MoAbs were able to recognize murine ChoKα (mChoK). Cell extracts from NIH3T3 fibroblast stably overexpressing mChoK (Tmar-9 and Tmar-11 cells) were resolved in an SDS-PAGE as described above. Although there is a 77% structure similarity among human and murine choline kinase (19), only a few of the generated MoAbs were able to effectively bind mChoK by WB (Fig. 1B, Table I).

Immunoprecipitation. Immunoprecipitation (IP) is one of the most widely used immunochemical techniques. In order to characterize the ability of the generated antibodies to immunoprecipitate ChoKα, Protein G-sepharose micro-beads were coated with the generated MoAbs. Immunoprecipitation of

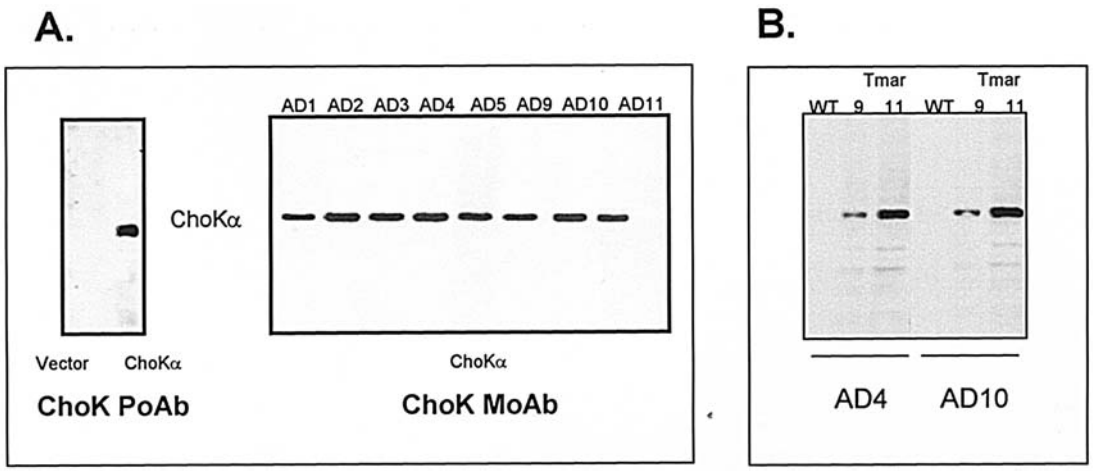

Figure 1. Characterization of generated monoclonal antibodies by Western blotting. Primary AD anti-ChoKα monoclonal antibody is indicated in each case. (A) Western blot of HEK293T cell extracts transfected with either pCDNA3 (negative control) or overexpressing ChoKα. As positive control, same extracts were used with the polyclonal anti-ChoKα serum (left panel). A unique band corresponding to the 53 kDa ChoKα protein is recognized by several representative monoclonals (right panel). All monoclonals were rather specific, with no detectable non-specific band. (B) Western blot of NIH3T3 extracts (WT), and NIH3T3 overexpressing mouse ChoKα (Tmar-9 and Tmar-11).

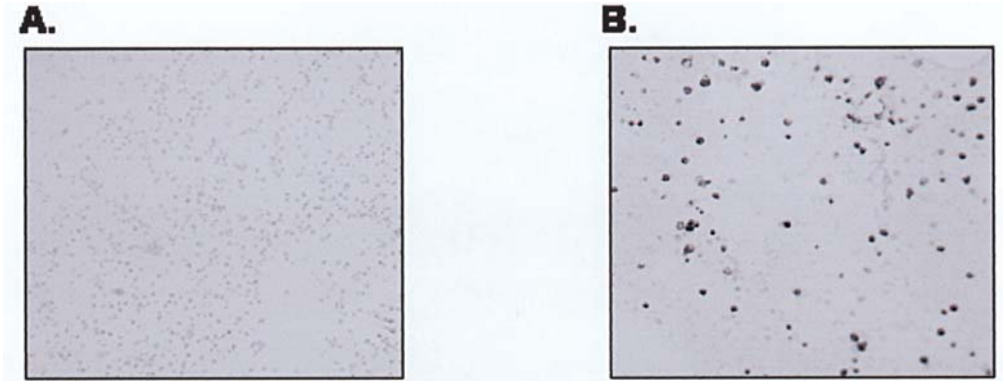

Figure 2. Cytoimmunostaining of ChoKα. Monoclonal antibodies were scored positive if positive for staining HEK293T cells transfected by ChoKα vector. AD2 was used for immunocitostaining of HEK293T transfected cells with the pCDNA3b empty vector (A), or ChoKα vector (B). A specific strong cytoplasmic staining of cells transfected with ChoK is shown.

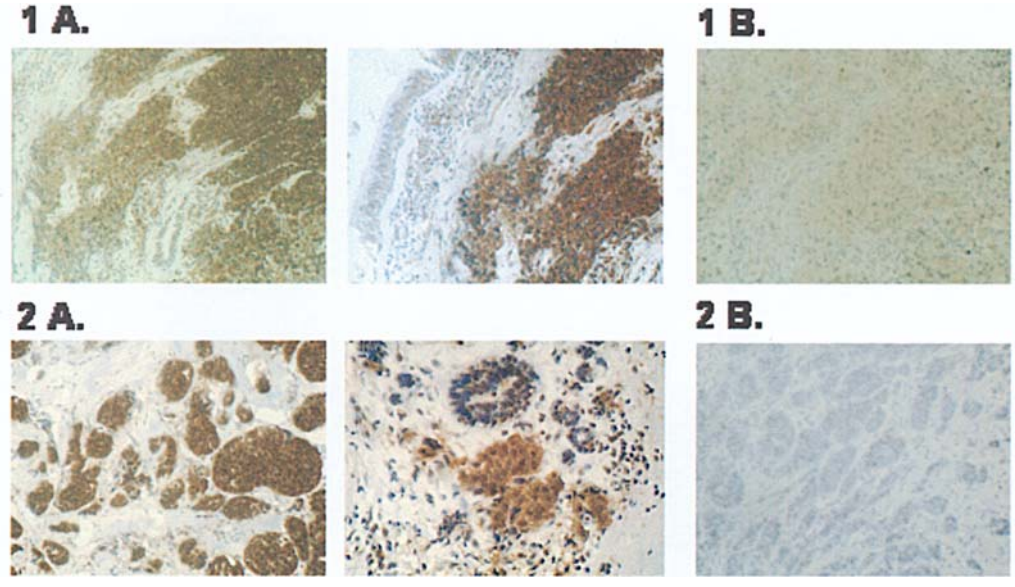

Figure 3. Identification of monoclonal antibodies to ChoKα useful for immunohistochemistry analysis. Immunohistochemistry analysis of lung (1) and breast (2) cancer tumors with representative anti-ChoKα MoAbs. Tumor tissues appear strongly stained with low or non-labelling in the adjacent normal tissues (A). ChoKα is not overexpressed in all tumor samples, a negative staining of each tumor type is presented (B). Counterstaining with haematoxylin.

ChoKα was performed from transfected HEK293T cell lysates as described above. The relative amount of ChoKα immunoprecipitated by each antibody was determined using an *in vitro* radioactive enzymatic activity assay based on the phosphorylation of choline to render o-phosphocholine (PCho) as previously described (12). Samples were resolved by a thin layer chromatography (TLC) and [^{14}C]-PCho was quantified. A total of 7 of the MoAb generated efficiently immunoprecipitated ChoK and the immunoprecipitated enzyme was fully active. However, the interaction of some of the antibodies with ChoKα could interfere with its enzymatic activity and although able to efficiently immunoprecipitate ChoKα, scored as negative in this enzymatic assay. Thus, the ability to immunoprecipitate ChoKα was also determined by Western blot using the polyclonal antibody in those MoAbs that scored negative in the enzymatic assay (data not shown). As summarized Table I, AD3 and AD8 MoAbs, and AD11 to a lesser extent, were able to immunoprecipitate ChoKα. These results suggest that some of the MoAbs may interfere with the enzymatic activity of ChoKα, resulting in a lack of ChoKα activity in the immunoprecipitates.

Immunohistochemistry. The potential use of the generated antibodies for immunohistochemistry (IHC) was analyzed. Monoclonal antibodies were first tested in paraffin-embedded cells that overexpressed ChoKα under optimal experimental conditions. To ensure high concentrations of the antigen, HEK293T cells were transfected with the plasmid encoding ChoKα, and as a negative control the pCDNA3b empty vector was used. Cell pellets of transfected HEK293T were embedded in liquid paraffin and processed as indicated in Materials and methods. An example of cytoimmunostaining performed with one of the MoAb generated with a strong and specific cytoplasmatic labelling in the HEK293T/ChoK transfected cells is shown in Fig. 2. These results indicate that the generated MoAbs were able to recognize ChoKα also in paraffin-embedded cells, and therefore show a strong affinity for ChoKα.

Since ChoKα has been recently described as a novel oncogene (4) with high incidence in human cancer (5-7), we tested the potential use of the generated MoAbs in cancer diagnostic by IHC analysis in human samples. Thus, an IHC ChoKα staining was performed in a few biopsies from patients previously diagnosed with breast cancer (Fig. 3). The analysis of stained biopsies reveals a strong labelling of some of the tumor tissues, whereas a faint staining is observed in adjacent normal tissue. However, in some biopsies both tumor cells and normal tissues show basal ChoK staining. Similar results were obtained when anti-ChoKα immunostaining was performed in biopsies of lung cancer, including NSCLC and SCLC samples (Fig. 3). These results are in keeping with our previous studies where 40-60% of breast, lung, colon and prostate tumors showed increased ChoK levels or activity. Similar results were obtained with 10 MoAbs, while 4 were negative in IHC analysis (Table I).

Discussion

Choline kinase α is a metabolic enzyme involved in the synthesis of phospholipids. A strong implication of ChoKα in cell proliferation, transformation and tumor progression has been reported (4,16). Using a polyclonal antibody by Western blot techniques, or *ex vivo* enzymatic analysis, overexpression of ChoK has been implicated in human carcinogenesis in breast, lung, colon and prostate tumors (5,6). These biochemical discoveries suggest that ChoKα could be a potential tumor marker in a wide variety of human cancers (5,6). We developed a set of monoclonal antibodies against ChoKα, and checked their usefulness in the diagnostics of human cancer using a standard IHC assay. We demonstrate that the MoAbs generated show high specificity and sensitivity against the antigen. Furthermore, these monoclonal antibodies to ChoKα constitute a useful and reliable tool for applications in basic research techniques such as Western blot and immunoprecipitation, that could be excellent tools to enrich our knowledge on ChoKα biology. Immunoprecipitation characterization of the MoAbs revealed the existence of different recognizing epitopes along the ChoKα structure, some of which could interfere somehow with its enzymatic activity. The availability of antibodies that interfere or keep intact the ChoKα activity could be very useful for a better understanding of its regulation. Moreover, since a novel antitumoral strategy using specific inhibitors of ChoKα has been demonstrated, MoAbs could be very important tools as specific inhibitors, or essential for monitoring of ChoK activity assays.

The results obtained in IHC assays are very encouraging for the application of ChoKα MoAbs in cancer diagnosis, prognosis and response to treatment. Biopsies from patients previously diagnosed of breast or lung cancer, were probed against immuno-ChoKα staining. In an extensive number of cases, this technique allowed us to clearly distinguish between tumoral tissue, where ChoKα is overexpresed and appears strongly labelled, from surrounding normal tissues with a faint anti-ChoK staining. Furthermore, ChoKα MoAbs were able to identify single tumor cells embedded into normal tissue in diffuse breast carcinomas with high reliability (data not shown). Previous biochemical analysis of overexpression and increased activity of ChoKα in human tumors showed that the incidence of ChoKα ranges from 40% to 60% in several human cancers (5-7). Thus, basal staining of tumors where levels of ChoKα are similar to those of normal surrounding cells would be expected. Indeed, several biopsies showed no difference between normal and tumor tissues, both scoring as negative by IHC. Therefore, the results obtained in IHC analysis confirmed previously reported evidence of ChoKα overexpression in human tumors.

Our results suggest that MoAbs against ChoK are useful tools for the identification of tumor cells by IHC. This implies a significant progress in the evaluation of ChoK overexpression and will allow the use of these MoAbs to investigate the relevance of ChoK overexpression in a large variety of human tumors. The generated MoAbs could be useful for IHC techniques in cancer diagnosis, prognosis or evaluation of response, both in retrospective and prospective studies.

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