Summary

Thymidylate synthase (TYMS), the critical enzyme for DNA synthesis and a target for chemotherapy, was recently characterized as an oncogene and a potential target for specific immunotherapy. Here we report TYMS-specific antibody response in a fraction of colon cancer patients. Humoral immune response to TYMS is induced by chemotherapy using TYMS inhibitors, such as 5-fluorouracil (5-FU), and may be associated with tumor burden. Therefore, TYMS may serve as a useful serological biomarker for monitoring the course of disease and treatment in cancer patients.

Keywords: Antibodies; Serology; Thymidylate synthase (TYMS); 5-Fluorouracil (5-FU)

1. Introduction

Thymidylate synthase (TYMS) is an enzyme essential for DNA synthesis and repair. TYMS is a homodimer that catalyzes the conversion of deoxuryridylate (dUMP) to deoxythymidine-5′-monophosphate (dTMP), which is further phosphorylated to become direct precursor for DNA synthesis—deoxythymidine-5′-triphosphate (dTTP) (for a review, see ref. [1]). TYMS protein and mRNA levels are often elevated in tumors and correlate with poor prognosis for cancer patients [2]. However, it remains unclear whether the increase of TYMS expression is caused by tumorigenic processes or whether the TYMS overexpression itself promotes aberrant cell growth and proliferation. Recently, oncogene-like features of TYMS have been reported: overexpression of TYMS in immortalized cells resulted in neoplastic transformation, characterized by increased proliferation and acquired tumorigenicity in nude mice [3].

Inhibition of thymidylate synthase leads to suppression of cellular growth or to cell death. For decades the TYMS inhibitor 5-fluorouracil (5-FU) has been successfully used in cancer chemotherapy, especially for gastrointestinal cancers [4]. The main limitation of 5-FU-based chemotherapy is the acquired resistance of tumors to treatment, caused, at least in part, by TYMS overexpression. There are different mechanisms by which 5-FU can increase levels of TYMS. In particular, free TYMS was reported to bind to its mRNA
providing negative feedback for its own biosynthesis. 5-FU disrupts this interaction leading to TYMS overexpression [5]. Amplification of the TYMS gene and accumulation of mutations, leading to increased stability of the TYMS protein after treatment of tumor cells with 5-FU, were also reported [6,7]. Thus, attempts to inhibit TYMS by 5-FU may cause TYMS overexpression and unresponsiveness of tumors to further treatment.

The increased expression level and occurrence of mutations of TYMS in 5-FU-resistant tumor cells make thymidylate synthase a speculative target for active specific immunotherapy in patients unresponsive to 5-FU based chemotherapy. Indeed, cytotoxic T-cells (CTLs) recognizing TYMS-derived peptides could be generated in vitro and were also detected in tumor-infiltrating lymphocytes of a colon cancer patient [8,9].

In this study we identified TYMS as tumor-associated antigen by serological screening of recombinant expression libraries (SEREX) [10,11]. We found increased titers of TYMS-specific antibody in the sera of colon cancer patients who received prior 5-FU chemotherapy and we also observed a putative association of humoral immune response to TYMS with the presence of tumors. Our data suggest that TYMS is a useful serological biomarker for monitoring status of cancer patients during treatment.

2. Materials and methods

2.1. Specimens

Patients’ sera were collected at the N.N. Blokhin Cancer Research Center (Moscow, Russia), Municipal Hospital # 24 (Moscow, Russia), and Krankenhaus Nordwest (Frankfurt, Germany). Normal sera obtained during routine diagnostic procedures were provided by the outpatient clinic of the Russian Ministry of Economics. Sera were aliquoted and stored at −80°C. To remove antibodies reactive with Escherichia coli and phage-related antigens, sera were pretreated as described earlier [12].

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

2.2. RNA extraction and construction of cDNA libraries

Total RNA from colon cancer cell line LoVo (provided by Dr. A. Gure, Ludwig Institute for Cancer Research) was extracted by TRI reagent (Sigma, Saint Louis, MO). Human testes total RNA was purchased from Ambion (Austin, TX). Poly A+ RNA was purified using the Oligotex mRNA Midi Kit (Qiagen, Valencia, CA). cDNA was prepared by ZAP Express cDNA synthesis kit (Stratagen, La Jolla, CA), ligated into the lambda ZAP expression vector and cloned using Gigapack III Gold Packaging Extract (Stratagene). Libraries containing 1–2 × 10^6 primary recombinants were amplified prior to immunoscreening.

2.3. Serological screening

Serological screenings of tumor-derived cDNA expression libraries (SEREX) and analysis of serological mini-arrays of recombinant tumor antigens (SMARTA) were performed as described earlier [13,14].

2.4. Purification of recombinant proteins

Hist6-tagged full-length TYMS and its fragments (Fig. 1C) were cloned into the pET-17b (TYMS and Fragments 1, 2) or pET-28b (+) (Fragments 3, 4) expression vectors (Novagen, San Diego, CA), expressed in E. coli Rosetta (DE3)pLysS cells (Novagen) and purified on Ni-NTA Agarose (Qiagen) in denaturing conditions according to the manufacturers’ recommendations.

2.5. Protein arrays

Proteins were spotted to the Hybond-C Extra nitrocellulose membrane (Amersham, Little Chalfont, Buckinghamshire, UK) 500–1 μg per spot. Arrays were dried, washed two times with Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST), blocked in TBS with 5% non-fat dried milk for 1 h at room temperature, incubated overnight at 4°C with pre-treated patients’ sera (according to ref. [12]) and then 1 h with alkaline phosphatase-conjugated goat anti-human Fcγ secondary antibodies, dilution 1:2000 (Jackson Immunoresearch, West Grove, PA). Arrays were developed with NBT/BCIP (Sigma).

2.6. Western blotting

Recombinant TYMS (2 μg per lane) was separated SDS-PAGE in 10% gel, transferred to a nitrocellulose membrane and incubated overnight with mouse monoclonal antibodies against Hist6-tag (Novagen 0.2 μg/ml) and TYMS (Lab Vision, Fremont, CA, Clone TS 106, 0.5 μg/ml) or with pre-treated human sera (dilution 1/500), washed and incubated for 1 h with HRP-conjugated goat-anti-mouse or goat-anti-human antibodies (Jackson Immunoresearch) in 1:10,000 dilution. Blots were developed using an enhanced chemoluminescence (ECL) Kit (Amersham).

3. Results

3.1. Identification of thymidylate synthase as serologically-defined cancer antigen

We and others used serological screening of tumor-derived cDNA expression libraries (SEREX) to identify
and characterize novel candidate tumor antigens expressed in several types of human cancers [10–16]. In this study we screened cDNA libraries derived from a colon cancer cell line and from normal human testis with pooled sera from colon cancer patients. We found positive clones corresponding to human thymidylate synthase in both libraries: clone MO-CO-1055 from LoVo-derived library and clone MO-TES-1055 from testis-derived library. Both clones reacted with the same sera, but clone MO-CO-1055 consistently showed somewhat brighter signal in serological analysis (Fig. 1A) and was used in most of subsequent experiments. It was molecularly defined as a partial transcript of human $TYMS$ gene lacking 182 bp of the complete cDNA at the 5′-end (Fig. 1C). The deduced amino acid sequence of MO-CO-1055 corresponded to $TYMS$ amino acid residues 26–313. No mutations in the coding region were detected in this clone as compared to human $TYMS$ gene sequence (reference sequence NM_001071).

To confirm further the reactivity of MO-CO-1055 positive sera with human thymidylate synthase, we performed Western blotting (Fig. 1B). Recombinant His6-tagged $TYMS$ was recognized by sera of MO-CO-1055/TYMS positive patients.

To identify the immunogenic regions in human thymidylate synthase we cloned a set of overlapping $TYMS$ cDNA fragments, expressed them as His6-tagged proteins and tested the recombinant proteins with serologically positive sera (Fig. 1C). We found that N-terminal region of $TYMS$ protein (aa 26–93) was recognized by sera of tested MO-CO-1055/TYMS positive patients.
Therefore, TYMS is an immunogenic protein, which can induce a robust antibody response in a fraction of cancer patients.

3.2. Antibodies to TYMS are found only in sera of colon cancer patients after 5-FU chemotherapy

To determine the frequency and specificity of immune response against TYMS, sera from normal individuals and patients with colon cancer were tested for their seroreactivity against clone MO-CO-1055 (Table 1). Interestingly, the most frequent serological response was observed in colon cancer patients who received chemotherapy regimens containing 5-FU. The frequency of anti-TYMS antibodies in primary colon cancer patients was extremely rare (Table 1). Importantly, antibodies to MO-CO-1055 were not detected in any of 360 normal donors’ sera tested (Table 1). Therefore, after serological analysis MO-CO-1055/TYMS emerged as a putative cancer-related antigen, specific for a fraction of 5-FU treated colon cancer patients.

3.3. Humoral immune response to TYMS correlates with the presence of tumor

To demonstrate a correlation of humoral immune response to TYMS with the status of disease we present a clinical case

<table>
<thead>
<tr>
<th>Patient CC224</th>
<th>Surgery</th>
<th>Chemotherapy</th>
<th>Disease status</th>
<th>TYMS antibodies</th>
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<tbody>
<tr>
<td>Surgery</td>
<td>Hemicolecotomy, cholecystectomy</td>
<td>Leucovorin, 5-FU</td>
<td>Multiple metastases in liver</td>
<td>Relative titer of TYMS antibodies</td>
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<tr>
<td>Chemotherapy</td>
<td>Hemihepatectomy</td>
<td>Eloxatin, Leucovorin, 5-FU</td>
<td>Partial regression of liver metastases, partial remission</td>
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<tr>
<td>Disease status</td>
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<td>Eloxatin, Leucovorin, 5-FU</td>
<td>No sign or symptoms of disease</td>
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Fig. 2. Clinical case of colon cancer patient CC224. Possible correlation of humoral immune response to TYMS with the presence of tumor. From top to bottom: treatments, course of disease, serology and quantification of antibodies to TYMS. Negative control—empty vector, positive control—SWAP-70. Quantification performed by program Image QuantaTM 5.2 (Amersham, Little Chalfont, Buckinghamshire, UK).
of a colon cancer patient with multiple metastases in gall bladder, liver and left lung who received chemotherapy including 5-FU (Fig. 2). The primary tumor was found to be positive for TYMS protein expression (data not shown). Histological evaluation of metastases was not performed, however, humoral immune response to TYMS completely disappeared after successful resection of metastases in left lung (Fig. 2). Thus, antibody response to human TYMS may depend on the presence of TYMS-positive tumor cells.

4. Discussion

5-Fluorouracil-based chemotherapy is the standard adjuvant treatment for colorectal cancer [2, 17]. 5-FU is usually applied in combination with several other drugs and such combined chemotherapy regimens increase median survival time to more than 20 months compared with 10–12 months if only 5-FU is used. However, the most effective 5-FU-based drug combinations induce responses in less than 60% of patients with metastatic colon cancer [4, 17]. At least one reason for unresponsiveness to 5-FU chemotherapy is the overexpression of TYMS in tumors [18] and in such cases alternative treatments are needed.

Active specific immunotherapy emerged as an effective treatment for gastrointestinal malignancies, especially for patients with minimal residual disease [19]. The suggestion of using TYMS as a target for immunotherapy appears as an alternative because high levels of TYMS expression in tumors are observed in about a half of colon cancer patients [2]. However, the density of TYMS-derived peptides on tumor cells must be high enough to make such therapy effective.

In previous studies TYMS was characterized as a tumor-associated antigen (TAA) and possible target for immunotherapy based on generation of cytotoxic T-lymphocytes (CTLs) against TYMS-derived peptides (aa 84–92, aa 117–125 and aa 251–259) in vitro [8] and on isolation of CTLs recognizing another set of TYMS-derived peptides (aa 189–198, aa 231–240 and aa 258–267) from tumor-infiltrating lymphocytes in colon cancer patient [9]. Here we observed specific antibodies reactive with TYMS in SEREX format of serological analysis that was also confirmed by Western blot analysis using recombinant TYMS protein. The main immunogenic region, responsible for serological recognition of TYMS, was mapped to N-terminus of TYMS with residual metastases in one colon cancer patient (Fig. 2) suggests that antibodies to TYMS may potentially become a useful marker of residual disease. Overall, in this study we identified TYMS as serologically-defined tumor-associated antigen, potentially useful for monitoring of cancer patients.

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References


