Complement Factor H as a Marker for Detection of Bladder Cancer

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Background: The BTA TRAK™ and BTA stat™ tests for bladder cancer use monoclonal antibodies (mAbs) X13.2 and X52.1 to detect factor H (FH)-related material in urine. The exact ligands remain unknown.

Methods: Western blot analyses of purified FH, recombinant factor H-related protein 1 (FHR-1), and serum and urine samples were used to identify the ligands of X13.2 and X52.1. Recombinant FH constructs were used to identify the target sites of X13.2 and X52.1. To analyze whether natural ligands of FH could compete with its recognition by the capture mAb X52.1, we used surface plasmon resonance analysis. The role of the ligands of X52.1 in the BTA TRAK assay was tested with use of purified proteins and FH-depleted samples.

Results: X13.2 bound to domain 3 of FH and FH-like protein 1, whereas X52.1 bound to domain 18 of FH and to FHR-1. Using specific FH depletion from a bladder cancer patient’s urine and purified FH, we demonstrated that FH is the ligand recognized by the BTA TRAK test. By contrast, FHR-1 in urine reduced the FH-dependent test signal.

Conclusions: FH is a tumor marker for bladder cancer. To reveal the presence of bladder cancer, the BTA TRAK assay detects FH, whereas FHR-1 is able to partly inhibit this detection. This indicates a special mechanism for a diagnostic immunoassay based on the combined effect of simultaneous positive and negative signals in a single sample.

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Bladder cancer (BC)6 is the fourth most common cancer and cause of cancer-related deaths in men and the eighth in women; the 5-year survival rate of BC ranges between 30% and 90% (1). The main reasons for the poor survival despite the availability of effective treatment are a high frequency of recurrence (50–80% depending on the initial stage and grade) and lack of a sensitive method for early detection, particularly carcinoma in situ tumors. BC is usually associated with hematuria, dysuria, and frequent urination, but more specific screening tests for BC have not been available until recently.

Several BC tumor markers have been identified recently (2–5). Among them, bladder tumor antigen (BTA) in urine is a sensitive marker and has been used as an adjunct to cystoscopy in the diagnosis and follow-up of patients with BC (6–10). BTA stat™ and BTA TRAK™ (Alidex) are 2 commercially available assays for BTA in urine. Both assays use 2 monoclonal antibodies (mAbs) that bind to complement factor H (FH)-related material isolated from urine of BC patients (11). The BTA stat is a qualitative cartridge-form enzyme immunoassay, and the BTA TRAK is a quantitative sandwich-type immunoassay in 96-well format. In both assays, the mAb X52.1 is used as the capture antibody and X13.2 as the detection antibody.

FH is a 150-kDa glycosylated plasma protein and the main inhibitor of the alternative pathway of complement. The concentration of FH in plasma is ~500 mg/L. It is also
peroxidase-conjugated rabbit anti-mouse and donkey anti-goat IgGs were from Jackson ImmunoResearch Laboratories Inc.

**PATIENT SAMPLES**

Standard midstream voided urine samples were obtained from 4 patients with histopathologically confirmed transitional cell carcinoma (TCC) of the bladder (Institutional Ethical Review Board acceptance #300896). After collection, the urine samples were immediately stored at −70 °C until the experiments were performed. The patient urine samples contained no detectable hemoglobin (<3 mg/L; reference interval, 0 to <10 mg/L). The normal human urine (NHU), normal human serum (NHS), and FHR-1-deficient serum (FHR-1-defS) were obtained from consenting Caucasian male laboratory workers.

**ELECTROPHORESIS AND WESTERN BLOTTING**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the standard procedure (23) with 10%, 12.5%, or 10–15% gradient gels under nonreducing conditions. For Western blots, the proteins were transferred from SDS-PAGE gels to nitrocellulose sheets (Schleicher & Schuell) (24), which were subsequently blocked with 50 g/L fat-free milk in phosphate-buffered saline. The membranes were incubated with either the mAb (2.5 mg/L) or polyclonal antibody (1:10 000 dilution) in the same solution for 1 h. After 3 washes with phosphate-buffered saline, the membrane was further incubated with peroxidase-conjugated antibodies at a dilution of 1:10 000 for 1 h. The bound conjugate was visualized by enhanced chemiluminescence substrate.

**RECOMBINANT CONSTRUCTS OF FH AND FHR**

A total of 9 recombinant constructs of human FH, in addition to recombinant FHR-1, were used in this study (Fig. 3). The cloning, expression, and purification with Ni-NTA resin of recombinant constructs consisting of SCRs 3–5, 1–4Δ3, 1–4Δ2, 1–4, 1–6, 1–7, and 8–20 of FH have been described elsewhere (22, 25). Constructs consisting of SCRs 18–20 and 19–20 of FH were cloned and expressed after amplification from a human liver cDNA library (Stratagene) with forward primers FH18f (5’-GA ATT CAA AGA GAC ACC TGT GTG-3’; restriction enzyme site underlined) and FH19f (5’-GA ATT CAA AAA GAT TCT ACA GGA AAA TGT G-3’) and reverse primer FH20r (5’-CCG CCG CTA TCT TTT TGC ACA AGT TGG-3’). The constructs were expressed in Pichia pastoris yeast strain X33 by use of expression vector pPICZa (Invitrogen) and standard techniques. The recombinant yeast proteins were purified to >95% by heparin affinity chromatography (Pharmacia). The construct FH18–20 was found to run as a doublet of bands in SDS-PAGE, and the upper band was verified as a glycosylated form by treatment with endoglycosidase H (Sig-
has previously been shown to be FH or a related protein molecule in both serum samples (Fig. 1). This molecule was confirmed as FHL-1 by use of recombinant proteins present in NHS and FHR-1defS. Both antibodies recognized an 150-kDa band present in all urines from patients with TCC. In one patient, in which the 43- and 37-kDa bands were not detected. The Western blot method is only semiquantitative, but by visual inspection of the band intensities, it appeared that the NHU sample required 50-fold concentration to obtain clearly detectable FH, FHL-1, and 110-kDa proteins in the binding assay were 100 mg/L for FH and 200 mg/L for X52.1 (27).

**Preparation of FH-depleted urine**
mAb 131X, which binds to FH but not to FHRs or FHL-1 (22), was coupled to CNBr-activated Sepharose [1 mg mAb/mL of wet Sepharose (Pharmacia)] according to the manufacturer’s protocol. To deplete FH from urine, we applied 2.0 mL of urine from a TCC patient to the column packed with 131X-coupled Sepharose; we collected the flow-through fractions and re-added them to the same column five times to achieve thorough depletion of FH. The efficiency of the FH depletion was monitored by Western blot using mAb 90X, which binds to SCR1 of FH and FHL-1 (22).

**RESULTS**

**Analysis of ligands for mAbs X13.2 and X52.1 in human serum**

To analyze the ligands for the mAbs X13.2 and X52.1 used in 2 commercially available TCC tumor marker tests, we first used the antibodies in Western blot analyses of NHS and FHR-1defS. Both antibodies recognized an ~150-kDa molecule in both serum samples (Fig. 1). This molecule has previously been shown to be FH or a related protein (11). In addition, X13.2 bound to a molecule of ~42 kDa present in both NHS and FHR-1defS (Fig. 1); this molecule was confirmed as FHL-1 by use of recombinant proteins in Western blots (Fig. 3). We therefore concluded that the ligands for X13.2 are FH and FHL-1.

In addition to binding to FH, the mAb X52.1 bound to two molecules of 37 and 43 kDa present in NHS but not in FHR-1defS (Fig. 1), indicating that the ligands for X52.1 are FH and the two previously characterized glycosylation variants of FHR-1 (FHR-1α and FHR-1β). Binding of mAbs X13.2 and X52.1 to FHR-1 was confirmed by use of recombinant FHR-1 in Western blots (data not shown). Neither of the antibodies bound to the other FHRs detectable in serum.

**Western blot of urine from patients with BC**

After analyzing the ligands of the tumor-marker-binding mAb in serum, we characterized the ligands in NHU and four urine samples from TCC patients. To obtain a detectable amount of FH in NHU, we concentrated the urine 50-fold, whereas the patient urines were used without concentration. All antigenically FH-related material in the urine samples was detected by polyclonal goat anti-FH antibodies (Fig. 2). In all patient urine samples, bands of 150 kDa (FH) and 42–43 kDa (FHR-1α and FHL-1) were detected. In addition, bands of ~37 (FHR-1β), 80, 110, and 300 kDa were detected in some samples. The 80- and 110-kDa bands were detected only under reduced conditions and are therefore likely to be fragments of FH.

The urine samples were used for Western blot with mAbs X13.2 and X52.1. As expected, mAb X13.2 bound to the 150- and 42-kDa bands in the urine samples from TCC patients and also to the ~300-kDa putative FH dimer band present in all urines from patients with TCC. In one of the patient urines, the 300-kDa band was the most prominent band.

mAb X52.1 bound to the 150-, 43-, 37-, and 300-kDa bands in all patient samples except in urine 3 from a TCC patient, in which the 43- and 37-kDa bands were not detected. The Western blot method is only semiquantitative, but by visual inspection of the band intensities, it appeared that the quantity ratio FH/FHR-1 was <1 in NHU, whereas it was >1 in all patient urine samples. The observation that the NHU sample required 50-fold concentration to obtain clearly detectable FH, FHL-1, and FHR-1 bands indicated that the amounts of FH and FHRs...
are much higher in the patient urine samples than in NHU, as expected on the basis of previous reports (11). It is noteworthy that the used unconcentrated NHU sample gave a negative result in the BTA-TRAK assay (the 50-fold-concentrated sample was not subjected to the assay).

**Mapping of mAb X13.2 and X52.1 Binding Sites on FH**

To investigate the reason that mAbs X13.2 and X52.1 bound to more than one ligand as well as the reason that the mAbs caused the previously reported differential effects on FH function (28), we mapped the binding sites of the mAb on FH by Western blotting using recombinant constructs of FH. The recombinant constructs used contained SCRs 1–7, 1–6, 1–4, 1–4Δ3, 1–4Δ2, 3–5, 8–20, 18–20, and 19–20 of FH (Δ represents a deletion). All constructs were recognized by a polyclonal antibody to human FH (Fig. 3, A and C). mAb X13.2 bound to plasma purified FH and the recombinant constructs FH1–7, FH1–6, FH1–4, FH1–4Δ2, and FH3–5, but not to the constructs FH1–4Δ3 or FH8–20 (Fig. 3B). Consequently, the binding site for X13.2 was mapped to SCR3 of FH. Because FHL-1 contains SCR1 through SCR7 of FH (29, 30), it is apparent that the binding site of X13.2 on FHL-1 is within SCR3.

**Analysis of the Effects of mAb X52.1 on the Interaction between FH and C3b**

It has previously been shown that mAb X52.1 does not inhibit cofactor activity of FH for C3b cleavage in the fluid phase (28). To test whether C3b or its fragment C3d could compete with X52.1 in binding to FH, we used a sensitive surface plasmon resonance analysis. mAb X52.1 did not have any inhibitory effect on the FH–C3d (Fig. 4) or FH–C3b interactions (data not shown). On the contrary, it enhanced binding of FH to C3d and C3b, probably by inducing dimerization of FH, as shown previously with other anti-FH mAbs (22). It is therefore evident that C3d and C3b do not compete with X52.1 in binding to FH and should not interfere with the diagnostic tests that use X52.1 as a capture antibody.

**Characterization of Ligands Recognized by the BTA Assays**

Both antibodies used in the BTA TRAK and BTA stat assays were found to have 2 ligands in plasma and urine;
Fig. 3. Mapping of binding sites of the anti-FH mAbs used in the BTA assays by Western blotting.

The purified recombinant constructs of FH (0.5 μg) were subjected to SDS-PAGE and Western blotting. The membranes were incubated with a polyclonal anti-FH antibody (A and C), mAb X13.2 (B), or mAb X52.1 (D). The SCR domains of each construct are indicated at the top of the corresponding lane. Mobilities of the size markers are indicated on the right and left.
however, only the ligands of the capture antibody could theoretically be recognized by an immunoassay. It therefore was necessary to analyze which of the ligands of mAb X52.1 (FHR-1, FH, or fragments of FH) are recognized in the patient sample by the diagnostic assays. We subjected increasing amounts of purified FH to the BTA TRAK assay and found a clear dose-dependent positive signal (Fig. 5A). To verify that FH is the recognized ligand, we specifically depleted FH from a TCC patient urine sample, using a third anti-FH antibody (131X) that binds to FH but not FHL-1 or FHR-1. This FH-depleted urine sample was subjected, in parallel with the original patient urine sample, to the quantitative BTA TRAK assay performed according to the manufacturer’s protocol. We found that although the non-FH-depleted patient urine sample gave a strong positive signal, the FH-depleted sample was clearly negative in the BTA TRAK assay (Fig. 5B). It therefore became evident that the ligand recognized by the BTA TRAK assay is FH.

The origin of FH in urine from BC patients has been suggested to be both local production by the tumor and leakage of plasma to the urine in combination with hematuria (11). It is known that several tumor cell lines produce FH and FHL-1 and that hematuria is not always present with carcinoma in situ-type bladder tumors that are recognized by the BTA assays. However, BC patients usually have hematuria; we therefore analyzed the effects of addition of diluted human serum to NHU (negative in the BTA stat and BTA TRAK assays). On addition of as low as 3 mL/L NHS to the urine, the BTA TRAK assay result became positive (Fig. 6A). The used 3 mL/L serum corresponds to ~6 mL/L (1:167) of blood contamination in urine. This is typical for moderate macrohematuria. We were able to detect low amounts of FH and FHR-1 in NHU samples (Fig. 2). These samples, however, were negative in the BTA TRAK assay. To determine the reason for this discrepancy, we considered whether FHR-1 could compete with FH in binding to the capture mAb in the BTA TRAK assay. Increasing amounts of FHR-1 (from 0.03 to 9.0 mg/L) were added to a buffer solution containing 0.3 mg/L FH, and the mixtures were analyzed by the BTA TRAK assay. FHR-1 reduced the signal in a dose-dependent manner down to 55%, at which point a 30-fold (by weight) excess (9 mg/L) of FHR-1 was present.
Discussion

In the present study, we have, for the first time, unequivocally shown that complement FH is the tumor marker in human BC and is recognized by both antibodies (X13.2 and X52.1) used in the BC tumor marker assays (BTA TRAK and BTA stat). Our results showed that mAb X13.2 binds to SCR3 of FH and FHL-1, whereas X52.1 binds to SCR18 of FH and to FHR-1. In addition, we showed that FHR-1 can compete with FH in binding to mAb X52.1 and that hematuria could cause positive results in the BTA TRAK TCC tumor marker assay. Our results indicate that although FH is the specific ligand recognized by the assay, FHR-1 can have an inhibitory effect on the measured values. This indicates that the assay uses the combined effect of simultaneous positive and negative signals in a single sample.

In the context of the diagnostic BTA TRAK or BTA stat assay, it is remarkable that C3b did not compete with mAb X52.1 in binding to FH. This indicates that the presence of C3b in urine samples should not have an effect on binding of the mAb to FH in the diagnostic procedure. In concordance with these results, it has previously been reported that mAb X52.1 actually enhances the strength of association of FH with C3b (28). In contrast, our finding that the presence of FHR-1 reduced the binding of FH to X52.1 in the BTA TRAK assay indicates that the presence of FHR-1 in a patient’s urine sample can affect the BTA TRAK test result.

The origin of FH in urine samples from TCC patients appears to be hematuria or production by the tumor cells. In blood or plasma, the amount of FHR-1 is significantly lower than that of FH (19). The amounts of FHR-1 and FH have not been studied extensively in normal urine, but on the basis of semiquantitative Western blots, it seems that the relative quantity of FHR-1 compared with FH is clearly higher than in plasma (Fig. 2). In addition, all tumor cell lines that have been shown to express FH produce only a minor amount of FHR-1, if any. In our analyses, all of the tested urine samples from TCC patients clearly contained less FHR-1 than FH (Fig. 2). It is therefore likely that the observed competition between FH and FHR-1 in the BTA TRAK assay does not compromise the sensitivity of the assay in clinical diagnosis of BC. On the contrary, because the relative quantity of FHR-1 compared with FH seems to be higher in normal urine than in plasma and because FHR-1 is not produced by the tumor cells, it is possible that the observed binding of X52.1 to FHR-1 might reduce the number of false-positive results in the BTA TRAK assay. However, FHR-1 could significantly inhibit the FH-dependent signal only in high concentrations (~50% reduction by 30-fold weight excess, i.e., ~90-fold molar excess). Because SCR18 of FH and SCR3 of FHR-1 are identical in amino acid sequences, it would be expected that the inhibition would be stronger. This discrepancy could be explained either by the possible effects of the neighboring domains (e.g., SCR17 of FH) on the availability of the epitope of X52.1 on SCR18 or by the fact that the FHR-1 used in the inhibition assay was a recombinant protein and therefore nonphysiologically glycosylated.

In conclusion, our results have revealed factors that can have an effect on the assay and therefore help in interpreting the results obtained for patient samples. On the other hand, this study provides a model in which a
diagnostic immunoassay simultaneously recognizes one molecule in a positive way and another molecule in a negative way.

We thank M. Ahonen for excellent technical assistance and Dr. John Tamerius for providing mAbs 90X and 131X. This work was supported financially by grants from the Academy of Finland (projects 201506 and 202529), the Helsinki University Central Hospital Funds, the Sigrid Jusélius Foundation, the Cancer Organizations of Finland, and the Finnish Cultural Foundation.

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