Immunooassay for Quantifying Squamous Cell Carcinoma Antigen in Serum

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BACKGROUND: Although the benefits of quantifying serum squamous cell carcinoma antigen (SCCa) have been reported, SCCa reagents were no longer available in the US by the late 1990s. Because SCCa quantification still has demonstrated clinical utility, we developed and validated a microtiter plate–based ELISA for measuring SCCa in serum.

METHODS: We coated microtiter strips overnight with capture anti-SCCa monoclonal antibody, washed the wells, added blocking buffer, and lyophilized the strips. For detection, we used a biotinylated anti-SCCa detection antibody, streptavidin/horseradish peroxidase conjugate, and tetramethylbenzidine/H2O2 substrate. A novel blocking reagent against human antimouse antibodies (HAMA) was evaluated. A reference interval was established with sera from healthy individuals and was confirmed in smokers.

RESULTS: The assay was linear to 40 μg/L SCCa (slope, 1.00; y intercept, 0.695; R², 0.996) with a detection limit of 0.3 μg/L. The intraassay imprecision results [mean (CV)] were 2.5 μg/L (3.4%), 18.0 μg/L (3.0%), and 30.7 μg/L (2.4%); interassay imprecision results were 2.0 μg/L (9.9%), 20.0 μg/L (7.6%), and 36.3 μg/L (3.5%). A correlation analysis against an established automated assay generated a slope of 0.976 and a y intercept of −0.193 μg/L (r² = 0.916). An upper reference limit of 2.1 μg/L SCCa was established at 95% confidence level, with no difference observed in smokers. No correlation between SCCa concentration and age was observed (r² = 0.0003). At a blocking reagent concentration of 5 mg/L, HAMA interference was eliminated in 3 samples known to produce falsely increased SCCa results.

CONCLUSIONS: This SCCa ELISA demonstrates acceptable performance characteristics for quantifying serum SCCa and is effective in eliminating HAMA interference.

Squamous cell carcinoma antigen (SCCa),4 a glycoprotein with isoforms ranging from 45 to 55 kDa, was first isolated from squamous cell carcinoma tissue of the uterine cervix (1) and may function as a protease inhibitor (2). It is produced in healthy epithelium and epithelial tissues (1, 3), and the isoforms consist of >10 proteins with pl values ranging from 5.9–6.6. These proteins are divided into 2 groups: the acidic group (pl <6.25) and the neutral group (pl ≥6.25) (4). Neutral SCCa proteins typically remain inside the cell, whereas acidic SCCa proteins are easily released and frequently increased in the serum of patients with squamous cell carcinomas or nonmalignant squamous cell lesions (2, 4).

SCCa may be involved in the malignant behavior of squamous cell cancers by functioning in invasion and/or metastasis. Consequently, the serum SCCa concentration is used for monitoring various carcinomas, including those of the uterine cervix, lung, skin, head and neck, esophagus, urothelium, anal canal, and vulva (3, 5–10). For example, serum SCCa was found increased in 72% and 94% of patients with confirmed cervix and lung squamous cell carcinomas, respectively (5, 8). Additionally, associations between serum SCCa concentrations and tumor stage, size, and progression have been observed in squamous cell carcinomas of the cervix and esophagus (7, 9, 11). Increased serum SCCa values have also been linked with benign diseases, including sinonasal inverted papillomas, ovarian cystic teratomas, and various skin disorders (12–15).

Despite the demonstrated clinical utility of SCCa testing, commercial assays became unavailable in the US by the late 1990s. Therefore, we developed and validated a microtiter plate ELISA as a practical alternative for SCCa testing. The assay includes a particularly effective reagent for protection against interference by human antimouse antibodies (HAMA).

Anti-SCCa monoclonal antibodies and stock SCCa were acquired from Abbott Japan Co. The antibodies (clones F2H7C1131 and F1H3C1151) were previously characterized by Nustad et al. (16), and were the same as those incorporated into Abbott’s automated SCCa assays used extensively in Europe and Asia. Microtiter strips (F8 MaxiSorp) were purchased from Nunc/Thermo Scientific. Biotin Protein Labeling Kits were obtained from Roche Diagnostics. Immunoglobulin Inhibiting Reagent® (IIR) was purchased from Bioreclamation. Streptavidin/horseradish peroxidase conjugate and Enhanced K-Blue 3,3′,5,5′-tetramethylbenzidine.

4 Nonstandard abbreviations: SCCa, squamous cell carcinoma antigen; HAMA, human antimouse antibodies; IIR, Immunoglobulin Inhibiting Reagent; TMB, 3,3′,5,5′-tetramethylbenzidine.
tetramethylbenzidine (TMB)/H₂O₂ substrate were acquired from BD Biosciences and NeoGen, respectively. The SpectraMax® PLUS plate reader (Molecular Devices) was controlled with the manufacturer’s SoftMax® Pro software.

Sera for assay development and from confirmed smokers (positive for nicotine and/or nicotine metabolites) were collected from patient samples that were sent to our laboratory for routine testing and had been deidentified under guidelines approved by the University of Utah Institutional Review Board. Samples were refrigerated at 4 °C to 8 °C for short-term storage (≤2 weeks) or frozen at −70 °C for long-term storage.

Blood samples from healthy volunteers were collected with the approval of the Institutional Review Board. Blood was collected by venipuncture into a serum separator tube, and the tube was inverted several times to mix the blood with the clot activator. The tube was then allowed to sit at room temperature for 30 min. After clotting, the sample was centrifuged at 1610g for 10 min to separate the serum from other blood components. The serum was then transferred to a 1-mL cryogenic vial and stored at −70 °C.

SCCa is highly concentrated in saliva and sweat (3). Therefore, face masks were worn throughout all procedures to prevent contamination of reagents and samples.

Microtiter strips were coated overnight at 4 °C with capture anti-SCCa monoclonal antibody (clone F2H7C1131; 2 mg/L antibody in 0.1 mol/L carbonate buffer, pH 9.6; 100 μL/well). The wells were washed once with wash solution (0.098 mol/L Na₂HPO₄, 0.009 mol/L KH₂PO₄, 0.137 mol/L NaCl, and 0.5 mol/L Tween 20, pH 7.2; 250 μL/well). Blocking buffer (0.008 mol/L Na₂HPO₄, 0.003 mol/L KH₂PO₄, 0.150 mol/L NaCl, 50 g/L sucrose, 20 g/L BSA, 0.5 mol/L Tween 20, pH 7.2; 250 μL/well) was added, and the strips were incubated 7–8 h with shaking (600–700 rpm) at room temperature. The strips were then lyophilized overnight at 4 °C to 8 °C and stored refrigerated in plastic bags with desiccant.

Anti-SCCa detection antibody (clone F1H3C1151) was biotinylated with the Roche Biotin Protein Labeling Kit. Eluent fractions were combined to produce an antibody concentration of approximately 0.5 g/L. Aliquots were stored at −70 °C.

Calibrators and controls were prepared by diluting SCCa stock with BSA/PBS solution (0.008 mol/L Na₂HPO₄, 0.003 mol/L KH₂PO₄, 0.150 mol/L NaCl, 10 g/L BSA, pH 7.2) to target values of 1, 5, 20, and 40 μg/L for the calibrators and to 2, 10, and 30 μg/L for controls. Aliquots (100 μL) were lyophilized and stored at −70 °C.

The ELISA was performed by reconstituting calibrators and controls with 100 μL distilled water. Calibrators, controls, and patient unknowns were then diluted 5-fold with BSA/PBS containing 100 mg/L IIR. This diluent was also used as the zero calibrator. We placed 100 μL of the diluted samples into the appropriate wells in duplicate. The plate was sealed with an acetate sealer and incubated 2 h at room temperature with shaking (600–700 rpm). After incubation, each well was washed manually 3 times with 250 μL wash solution. Biotinylated SCCa antibody was diluted (1 part in 3000 parts BSA/PBS), and 100 μL was added to each well. The plate was resealed and incubated with shaking for another 1 h. After a second wash, 100 μL of streptavidin/horseradish peroxidase conjugate (1 part in 200 000 parts BSA/PBS) was added to each well. The plate was resealed and incubated with shaking for 30 min. After a final wash, 100 μL TMB/H₂O₂ substrate was placed into each well, and the plate was incubated for 10 min with shaking. The reaction was stopped by adding 0.5 mol/L H₂SO₄ (100 μL/well). The absorbance of each well was measured at a single wavelength (450 nm); a calibration curve was constructed with a 4-parameter fit.

Comparison of the absorbances for controls and unknowns with the calibration curve generated SCCa concentrations expressed in micrograms per liter.

The assay was linear to 40 μg/L SCCa with a slope and y intercept of 1.00 (95% CI, 0.959–1.05) and 0.695 (95% CI, −0.073 to 1.460), respectively (R² = 0.996) (see Fig. 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol56/issue9). The limit of detection (0.3 μg/L) was evaluated as the mean of 10 replicates of the zero calibrator (0.07 μg/L) plus 2 SDs (SD, 0.12 μg/L). Mean (SD) intraassay results (n = 12) of 2.5 (0.08) μg/L, 18.0 (0.54) μg/L, and 30.7 (0.73) μg/L had intraassay CVs of 3.4%, 3.0%, and 2.4%, respectively. Interassay results (n = 10) of 2.0 (0.20) μg/L, 20.0 (1.52) μg/L, and 36.3 (1.28) μg/L had interassay CVs of 9.9%, 7.6%, and 3.5%, respectively.

We used 30 samples obtained from and assayed previously by Abbott with their IMx® SCCa assay to conduct a split-sample study. Deming regression of our ELISA vs the IMx assay generated a slope of 0.976 and a y intercept of −0.193 μg/L (r² = 0.916).

A reference interval was established with serum samples from 136 healthy individuals 19–75 years of age (66 males, 70 females). Nonparametric analysis at a 95% confidence level generated an upper reference limit of 2.1 μg/L SCCa. There was no correlation between age and SCCa concentration (r² = 0.0003; Fig. 1).

The 2.1-μg/L upper reference limit is in agreement with the limits identified in several other studies (approximately 2.0 μg/L) (5–7). This value also compares acceptably with data summarized in the IMx SCCa package insert, which indicates that 99% of healthy individuals (n = 885) have SCCa concentrations <2.5 μg/L (17).
Because the vast majority of our volunteers for establishing the reference interval were nonsmokers, we analyzed a population of strictly smokers. Results (n = 24) ranged from <0.3 µg/L to 2.9 µg/L with a mean of 0.6 µg/L and a 95% upper confidence limit of 2.1 µg/L. Because this value agrees with our established reference interval, we conclude that smoking status does not affect serum SCCa concentrations.

Because of the known effects of HAMA in immunoassays (18), HAMA protection was integrated into our assay. We chose the novel IIR because it is not simply precipitated IgG but rather a combination of murine monoclonal antibodies (Bioreclamation’s proprietary formula). We also chose IIR for its demonstrated superiority to other HAMA-blocking reagents (19, 20). Sera from 3 individuals with known HAMA concentrations of 11 300, 33 500, and 161 000 µg/L and known to produce falsely increased SCCa results in the absence of HAMA-blocking agents were treated with IIR at several concentrations. At an IIR concentration of 5 mg/L, HAMA was successfully blocked in all samples (Table 1). To increase the certainty that all HAMA effects would be blocked, we implemented an IIR concentration 20 times this value (100 mg/L) for the assay. Therefore, interference would be eliminated to a hypothetical limit of at least 20 times the highest HAMA concentration evaluated (20 × 161 000 µg/L, or 3.2 g/L).

To investigate any potential effects of IIR, we split each of 3 serum samples into 2 aliquots. One aliquot was assayed with sample diluent minus IIR, and the other was assayed with diluent containing 400 mg/L IIR, 4 times the 100-mg/L concentration used in the assay. No differences were observed (CVs <3.9% for all 3 aliquot pairs).

In conclusion, the SCCa ELISA described is a viable alternative that demonstrates acceptable performance for quantifying serum SCCa. The upper reference limit of 2.1 µg/L agrees with other published reference intervals used in SCCa testing (5–7, 17), with no difference observed between smokers and nonsmokers. In addition, the ELISA is effective in eliminating HAMA interference, thus minimizing the potential of falsely increased results.

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**References**


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