SUPPLEMENTARY METHODS

Detection of Anti-Conatumumab Antibodies

Serum samples were treated with acetic acid to dissociate antibody complexes and were incubated with a conjugate/neutralization mixture consisting of biotinylated conatumumab, ruthenylated conatumumab, and Tris buffer (pH 9.5) for 15 to 24 hours. The sample mixture was added to a MSD 6000 streptavidin microtiter plate (blocked with 1× PBS and 1% bovine serum albumin [BSA]), the plate was washed, and complexes consisting of anti-conatumumab antibodies bound to labeled conatumumab were detected using MSD T Buffer that could detect ruthenylated conatumumab through electrochemiluminescence (ECL). ECL was measured by a MSD 6000 plate reader (Meso Scale Discovery, Gaithersburg, MD) and quantified as ECL units. Serum samples with a signal/noise ratio above a validated cut point in this assay (1.23) were further analyzed in a specificity assay as follows. Samples were treated with a high concentration of conatumumab, and those that had a signal/noise below or equal to 1.23 or exhibited signal reduction more than 50% in the presence of excess conatumumab were reported as positive for anti-conatumumab antibodies.

Measurement of Serum Conatumumab Concentration

The serum concentration of conatumumab was determined by using a validated enzyme-linked immunosorbent assay (Amgen Inc., Thousand Oaks, CA). Briefly, standards and quality control samples (made by spiking conatumumab into 100% human serum) and study samples were loaded into microplate wells coated with a mouse anti-conatumumab monoclonal antibody

(clone #11C2, Amgen Inc.). Captured conatumumab was detected by using a biotinylated rabbit anti-conatumumab polyclonal antibody (Amgen Inc.), streptavidin-conjugated poly-horseradish peroxidase (Pierce Chemical Co., Rockford, IL), tetramethylbenzidine, and hydrogen peroxide. The optical density was measured at 450 to 650 nm, and data were reduced by using the Watson version 7.0.0.01 data reduction package using a 4-PL Logistic (auto estimate) regression model with a weighting factor of 1/Y.

Immunocytochemical Detection of Cleaved Caspase-3

Fixed tumor cells were pelleted by centrifugation, resuspended in PBS, and cytocentrifuged onto microscope slides. The cytospots were resuspended in 100 μL of PBS containing 1% BSA and 0.1% sodium azide (PBS-BSA), and blocked for 30 minutes with PBS-BSA containing 5% normal goat serum and 5% normal horse serum. The cells were then incubated overnight in the dark at 4°C with 100 μL of PBS-BSA containing 0.25 μg/mL mouse anti-EpCAM monoclonal antibody (clone VU1D9; EpCAM is a recognized marker for epithelial cells[31,32]). The slides were washed in PBS-BSA and incubated overnight in the dark at 4°C with 100 μL of PBS-BSA containing 0.2% Triton X (PBS-BSA-TX) and either anti-caspase-3 antibody (Asp175; Cell Signaling Technologies, Danvers, MA, catalog number 9661L) or a solution of anti-caspase3/blocking peptide (Cell Signaling Technologies, Danvers, MA, catalog number 1050). The cells were then incubated with 100 μL of PBS-BSA-TX containing secondary antibodies conjugated to Alexa488 and Alexa633 (Molecular Probes, Eugene, OR) for 1 hour in the dark at room temperature. Finally, the cells were stained with 100 μL Hoechst solution (2 μg/mL; Molecular Probes) and incubated for 10 minutes in the dark. The slides were mounted and the cells analyzed by Laser Scanning Cytometry.

Laser Scanning Cytometry

Cytometric measurements were performed by using an iCyte Laser Scanning Cytometer. The excitation wavelengths were 405 nm, 488 nm, and 633 nm, generated by the Violet, Argon, and HeNe lasers, respectively. Blue (Hoechst 33342), Green (Alexa488), and Long Red (Alexa633) fluorescence were measured by separate photomultipliers. Adjacent field images were scanned using 40× magnification, and the percentage of individual nucleated caspase-3–positive cells was determined. Cellular image quantification was based on nuclear thresholding, and populations were gated to exclude large clumps of cells. The activated caspase-3–positive population was identified on the basis of the intensity of the Alexa633 signal after exclusion of spectral overlap and comparison with control samples (secondary antibody alone and/or peptide-blocked primary antibody). The integrity of the population was verified by relocating positive events into an image gallery and visually confirming the morphology of positive stained cells. Tumor cells were identified on the basis of the intensity of the Alexa488 signal.