Published in final edited form as:

Appl Immunohistochem Mol Morphol. 2016 April ; 24(4): 246–252. doi:10.1097/PAI.0000000000000292.

Mesothelin expression in advanced gastro-esophageal cancer represents a novel target for immunotherapy

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Abstract

The identification of new therapeutic targets is of profound importance if we are to improve outcomes in gastro-esophageal cancer. This study assessed the rate of mesothelin expression in tumors of Western patients with upper gastrointestinal tract (GI) carcinomas. In addition, the AGS gastric cancer cell line was tested for sensitivity to SS1(dsFv)PE38 (1), a mesothelin-targeting immunotoxin. Previously constructed tissue microarrays containing samples from 127 patients with gastro-esophageal adenocarcinomas were examined by immunohistochemistry (IHC) for mesothelin expression. Labeling for HER2-neu, E-cadherin and c-Met were also assessed. Tumors were considered positive for mesothelin if at least moderate cytoplasmic/membranous or luminal staining was present in minimum 10% of the neoplastic cells. The AGS gastric cancer cell line was assessed for surface mesothelin expression by flow cytometry and the viability of cells treated with SS1P was measured. Gastroesophageal cancers were mesothelin positive in 64/127 tumors (50.4%, 95% CI: 41.4 to 59.4%) while only 9 carcinomas (7.1%, 95% CI: 3.3 to 13.0%) were Her2-neu IHC 3+ positive and 8 (6.6%, 95% CI: 2.9 to 12.5%) were c-met positive. Mesothelin expression increased from stage I to stage IV tumors (37.5% to 56.3% respectively, $p=0.10$). The AGS gastric cancer cell line was sensitive to the immunotoxin with an EC50 value in the low picomolar range (0.4 ng/mL). A gastric cancer cell line derived from a Western patient was exquisitely sensitive to the mesothelin-targeted immunotoxin SS1P. Clinical trials involving novel mesothelin targeted immunotherapeutics in gastro-esophageal cancer are currently in development.

Keywords

carcinoma; expression; gastric; gastro-esophageal; immunotherapy mesothelin; target

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Disclosure/Conflict of Interest

A device used in the study described in this article is manufactured by Ventana Medical Systems. The Illei has served as a consultant to Roche Diagnostics/Ventana Medical Systems. This arrangement has been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies. The other authors declare that they have no conflict of interest.

The development of targeted agents for gastric and gastro-esophageal carcinomas has been limited when compared to other common tumors. Palliative chemotherapy is often given to patients with metastatic gastro-esophageal carcinomas, and the median survival remains less than one year(2). To date trastuzumab, in combination with chemotherapy, is the only approved novel agent for these patients. Its use is however limited due to the small population of patients whose tumors over-express HER-2 (18%-22%)(3). High density genomic profiling arrays analyzing somatic copy-number alterations in 296 gastro-esophageal cancers have identified amplified genes in 37% of these cancers, most notably, amplifications of *ERBB2*, *FGFR1*, *FGFR2*, *EGFR* and *MET*(4). The vascular endothelial growth factor receptor-2 (VEGFR-2) antagonist ramucirumab has recently demonstrated modest activity in patients with advanced gastro-esophageal cancer that has progressed after first line therapy(5). Additional druggable targets that occur in a higher percentage of patients are required if we are to improve patient outcomes.

Mesothelin is a 40-kDa cell surface glycoprotein that is present on normal mesothelial cells lining the pleura, peritoneum and pericardium, surface epithelial cells of the ovary, tunica vaginalis, rete testis and the tonsillar and fallopian tube epithelial cells(6) (7). Mesothelin was originally identified as the antigen recognized by the monoclonal antibody (mAb) K1 that was produced by immunization of mice with the human ovarian cancer cell line OVCAR3(8). Mesothelin is highly expressed in several human tumors, including epithelioid mesotheliomas, pancreatic/biliary adenocarcinomas (~100% of cases) and in ovarian carcinomas (~70% of cases)(7, 9-12). In the majority of these cancers there is diffuse, cell surface or luminal mesothelin expression. The prevalence and characterization of mesothelin expression in different tumor types has been described in prior reviews(13) and mesothelin is commonly expressed in Barrett's esophagus associated esophageal adenocarcinoma(14). Two recent studies from Japan have identified mesothelin expression in 45%-59% of resected gastric cancer specimens but expression in patients with gastric cancer from a Western population have not been assessed(15, 16).

Our study was conducted to determine the prevalence of mesothelin expression in gastric cancers from a population of Western patients. We also evaluated the sensitivity of the AGS gastric cancer cell line to SS1(dsFv)PE38 (1), a mesothelin-targeted immunotoxin. SS1P binds specifically to cells that express mesothelin, and the subsequent uptake and processing by the cell results in intracellular release of the toxin. Inside the cell, the toxin halts protein synthesis, inducing cell death. The toxin is unable to kill cells that do not express mesothelin. It was anticipated that high mesothelin expression in gastric cancers and limited expression on normal tissue would make it an attractive tumor associated antigen for cancer therapy in a tumor type with limited identifiable oncogenic driver mutations.

Materials and Methods

This study was performed with the approval of the Institutional Review Board of the Johns Hopkins University. The subjects of this study were 127 patients who underwent surgery for gastric or gastro-esophageal junction (GEJ) adenocarcinoma between April 1985 and January 1992. Immunohistochemistry (IHC) for mesothelin, Her2/neu, c-met and E-cadherin

was performed using previously constructed tissue microarrays (TMA) containing 116 gastric and 11 GEJ adenocarcinomas (See Table 1). The TMA contain at least two 1 mm cores of primary tumor, samples of benign stomach, duodenum and in a subset of tumors matched regional lymph node metastases.

IHC for mesothelin (clone 2C6, Aduro BioTech, Inc., Berkeley, CA) was performed using a previously standardized protocol (unpublished) on a Leica Bond autostainer (Leica Biosystems, Buffalo Grove, IL) with a biotin free polymer detection (Bond Polymer Refine Detection, Leica Biosystems, Buffalo Grove, IL). To determine what antibody to use we tested one commercial (clone 5B2 Vector Laboratories, Burlingame, CA) and two non-commercial (clone D4038 & 2C6, Aduro BioTech, Inc., Berkeley, CA) mouse monoclonal anti-mesothelin antibodies on previously constructed tissue microarrays (TMA) containing 33 ovarian papillary serous carcinoma, 37 pancreatic adenocarcinomas, and 24 (22 epithelioid, 2 biphasic) malignant mesotheliomas (Table 2) and the antibody providing the strongest labeling and the cleanest background was selected (clone 2C6)

Her2/neu expression was detected using the Herceptest (DAKO, Carpinteria, CA) and an FDA approved method with a semi-automated pretreatment chamber (PT-Link, Dako, Carpinteria, CA) and autostainer (Dako, Carpinteria, CA). The E-cadherin and cmet immunolabeling (E-cadherin rabbit monoclonal antibody EP700Y, c-met rabbit monoclonal antibody SP44; both Ventana Medical Systems, Tucson, AZ) were performed using a Ventana Benchmark XT automated system and a biotin free detection kit (UltraView, Ventana Medical Systems, Tucson, AZ). The immunolabeling was evaluated for extent and intensity by two of the authors (PBI and MLC) for all four antibodies. The Herceptest slides were evaluated according to established criteria for resection specimens of gastric tumors(17, 18). Stains for mesothelin and E-cadherin were evaluated for cytoplasmic, membranous and luminal labeling using a 4-tier semi-quantitative scoring system (Table 3) similar to one we used in a prior study(19). The final score represents a combination of all labeling patterns and an average of the tumor cores. Cases were considered mesothelin or E-cadherin positive if at least moderate cytoplasmic, membranous or luminal labeling was present in 10% or more of the tumors cells (Figure 1) (20-22). Labeling for c-met was considered positive if at least 50% of the tumor cells exhibited moderate and/or strong membranous staining similar to scoring criteria proposed for non-small cell lung carcinomas(23, 24). The tumors were grouped into low positive or high positive for all markers based on strong labeling present in less (low) or more (high) than 50% of the tumor cells.

Cell Culture and reagents

The AGS cell line was obtained from ATCC and recently authenticated by STR testing. Cells were grown at 37°C with 5% CO₂ in RPMI1640 medium (Lonza) supplemented with 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U penicillin, 100 µg streptomycin (Gibco Life Technologies) and 10% fetal bovine serum (FBS) (HyClone, Thermo Scientific). Immunotoxin SS1P was manufactured by Advanced BioScience Laboratories, Inc. as previously described(25).

Mesothelin Surface Expression Assay

The gastric cancer cell line AGS was assessed for surface mesothelin expression by flow cytometry. A minimum of 20,000 live cells per sample were analyzed on a FACS Calibur (BD Bioscience) running CellQuest software (BD Bioscience). Data were processed using FloJo software (Tree Star, Inc., Ashland, OR). Cells were plated at sub-confluent density and grown for 48-96 hours in culture before detachment with trypsin. All subsequent steps were performed at 4°C. Live cells were washed, re-suspended in FACS buffer (5% FBS, 0.1% sodium azide in DPBS without calcium or magnesium) and then incubated with mouse anti-mesothelin antibody (MN, Rockland Immunochemicals Inc., Gilbertsville, PA) for 30 minutes. Cells were washed again and incubated for 30 minutes in the dark with PE-conjugated secondary antibody (RPhycoerythrin-Conjugated AffiniPure (Fab')² Fragment Goat Anti-Mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA). Geometric means were chosen as mean fluorescence intensity (MFI). The MFI of cells was compared with the MFI from a standard curve of PE-conjugated calibration beads (BD QuantiBRITE™ PE quantitation kit, BD Bioscience) and the number of mesothelin sites per cell was estimated.

In Vitro Assays of Immunotoxin SS1P on Gastric cancer cells

The viability of gastric cancer cells treated with the immunotoxin were measured using the Cell Counting Kit-8 WST-8 assay (Dojindo Molecular Technologies, Inc.). Cells (1.0×10^4 /well) were seeded in 96-well plate and incubated at 37°C for 4-6 hours before treatment with 0.01, 0.1, 1.0, 10 and 100 ng/mL of SS1P in complete medium, then incubated at 37°C for another 72 hours. WST-8 assay reagent was added per manufacturer's instructions, plate was incubated at 37°C, and absorbance at 450 nm was measured. Values were normalized between 0% viability for treatment with cyclohexamide (10 µg/mL) which produces complete cell killing and 100% for addition of complete medium. Each immunotoxin concentration was tested in triplicate for each experiment. Curve fits and IC₅₀ for each experiment were calculated using Prism software non-linear regression fit for log(inhibitor) vs. normalized response.

Statistics

The primary statistical objective of this study was to estimate the proportion of upper GI tumors from patients from the United States that were positive for mesothelin expression and to determine if expression of mesothelin correlated with stage of disease. Secondary objectives were to similarly assess expression of E-cadherin, HER2/neu, and c-met, as well as compare mesothelin expression by location and Lauren classification. Proportions are reported with exact binomial 95% confidence intervals. The Cochran-Armitage test for trend, or its exact version, was used to determine if the probability of positive expression increased with increasing stage of disease. The associations of tumor location and Lauren classification with positive expression were assessed with Chi-square or Fisher's exact tests as appropriate. All P-values reported are two-sided, and the significance level was set at 0.05 for all analyses. Statistical analyses were performed using R version 3.00.

Results

Mesothelin positivity was seen in 64 of 127 adenocarcinomas (50.4%) while only 9 (7%) were Her2/neu positive (IHC 3+). The study sample consisted primarily of gastric adenocarcinomas 116 (91%) and 11 (9%) gastroesophageal junction (GEJ) adenocarcinomas (as determined on resection specimens) of which 5 (45%) were mesothelin positive and 6 (55%) mesothelin negative.

There was a trend of increasing mesothelin expression with stage of disease. Positive expression was identified in 9 (37.5%) of the 24 stage 0 (Tis) or stage I, in 14 (45.2%) of the 31 stage II, in 23 (57.5%) of the 40 stage III, and in 18 (56.3%) of the 32 stage IV adenocarcinomas ($p=0.10$, trend test). Gastric adenocarcinomas were also classified according to the Lauren classification as either diffuse, intestinal or mixed subtypes. In this analysis 22/48 (46%) of diffuse gastric cancers were mesothelin positive and 34/61 (56%) of intestinal type gastric cancers were positive. Mesothelin expression did not correlate with location, Lauren classification or gender.

In the TMA-s we included matched normal gastric and duodenal mucosa for most tumors and benign esophageal mucosa for a selected number cases (primarily GEJ adenocarcinomas). No mesothelin labeling was seen in duodenal mucosa, and 91% of gastric and 92% of esophageal mucosa samples were also negative. The majority of positive gastric mucosa samples showed focal weak cytoplasmic labeling (7.2% of total) with only rare samples showing more diffuse cytoplasmic labeling (1.8% of total). Similarly weak cytoplasmic labeling was seen in the positive esophageal samples (8% of total). No strong labeling or luminal labeling was seen. Evaluable tissue of matched lymph node (LN) metastases was available for 68 adenocarcinomas. Of these 68 adenocarcinomas, 44 (66%) had identical scores for mesothelin in both primary tumor and LN metastases. An additional 9 were positive in both samples but had stronger labeling in the primary tumor (3+ vs. 2+) while one case had weaker labeling in the primary tumor (2+ vs. 3+) resulting in a total number of matched positive cases to 53 out of 68 (78%). Of the remaining 15 cases, 12 had no labeling of the metastases and variable staining of the primary tumor (1+: 5; 2+: 5; 3+: 2), the remaining 3 cases had 2+ labeling of the primary tumor and 1+ labeling of the metastasis. Our expression results are comparable to those seen in an Asian population.

Her2/neu positive expression was detected in 9 of 127 (7.1%, 95% CI: 3.3 to 13.0%), E-cadherin in 115 of 126 (91.3%, 95% CI: 84.9 to 95.6%), and c-met in 8 of 122 (6.6% 95% CI: 2.9 to 12.5%) adenocarcinomas. The expression of Her2-neu was positively correlated with stage ($p=0.05$, exact trend test) while E-cadherin and c-met expressions were not correlated with stage of disease ($p=0.38$ and $p=0.40$, exact trend tests respectively). In 58 adenocarcinomas with diffuse or mixed histologic type (Lauren classification), 1 (1.7%) was identified as Her2-neu positive, while 8 (11.8%) of 68 intestinal type tumors were positive for Her2-neu ($p=0.04$). This reinforces the need to identify new targets in patients with diffuse gastric cancer who will not benefit from HER2/neu targeted agents.

In order to determine whether gastric cancer expresses enough surface mesothelin to be successfully targeted using mesothelin targeted therapy, we examined surface expression

levels of mesothelin in the AGS cell line (derived from a North American patient) using FACS analysis (Figure 2). We found that these cells had strong expression with an average of 8.8×10^3 ($\pm 1.6 \times 10^3$) mesothelin binding sites/cell as determined in triplicate assays. AGS cells were then tested for their sensitivity to SS1(dsFv)PE38 (1), a mesothelin-targeted immunotoxin, using a cell viability assay (Figure 2). We found that AGS was exquisitely sensitive to the immunotoxin with an average EC50 value in the low picomolar range ($0.4 \text{ ng/mL} \pm 0.1$, over $n = 6$ experiments). These results demonstrate that some gastric cancers do express sufficient levels of mesothelin to be effectively killed by mesothelin-targeted therapies like the SS1P immunotoxin.

Discussion

In this study we report that mesothelin is highly expressed in gastric and gastroesophageal junction adenocarcinomas in Western patients and that expression increases from early stage to more advanced stage. We also found that a novel mesothelin-targeted immunotoxin SS1P has extremely promising preclinical activity in a gastric cancer cell line derived from a Western patient. At the present time there is no universally accepted scoring system to determine mesothelin expression. We chose a 4-tier semi quantitative scoring system that we have used in a prior study to see if stronger/more diffuse staining has significance. We also included cytoplasmic, luminal and membranous staining together rather than evaluating each pattern separate and based on our prior experience and published data we choose a threshold of a minimum of 10% tumor staining for positivity(19-22). We included matched normal gastric and duodenal mucosa when evaluating the tumors and determined that in over 90% of the cases that the normal tissue did not express mesothelin. The majority of the mesothelin positive gastric mucosa samples showed focal weak cytoplasmic staining (7.2% of total) with only very rare samples showing more diffuse cytoplasmic staining (1.8% of total). No strong labeling or luminal staining was seen in normal tissue which is of considerable importance for future immunotherapeutic targeting strategies.

We were also able to evaluate tissue of matched lymph node metastases for 68 tumors. The vast majority of these tumors 53/68 (78%) had either identical scores for mesothelin in both primary tumor and lymph node metastases or slightly stronger labeling in the primary tumor. At the present time we do not know if the intensity of labeling matters or will impact on therapeutic efficacy in future clinical trials. Here we found that 50% of gastric carcinomas were considered mesothelin positive with 24% determined as low positive (IHC 2+) and 26% as high positive (IHC 3+) with stage IV disease having more mesothelin expression (56%) than stage I (37%).

Mesothelin has been proposed as a target of interest for antibody-based therapies in solid tumors most notably to date in malignant mesothelioma, ovarian and pancreatic adenocarcinomas(19, 26). Early phase clinical trials investigating immunotherapeutic agents have shown that targeting mesothelin is safe and does not result in enhanced toxicity to normal tissue(19). Herein we show preclinical data indicating that mesothelin targeting in gastric cancer is a potentially very promising strategy. The gastric cancer cell line AGS is exquisitely sensitive to the immunotoxin SS1P with EC50 values in the low picomolar range (0.4 ng/mL). In clinical trials performed to date in mesothelioma, blood levels of SS1P of

500-1000 ng/ml can be achieved without dose limiting toxicity(1). One factor that has limited the activity of large-molecular weight molecules like immunotoxins and antibody drug conjugates is their degree of penetration into solid tumors. This can be abrogated by combining them with chemotherapy which has the dual effect of disrupting tumor packing and lowering intra-tumoral fluid pressure(27).

An interesting therapy that could be evaluated clinically in advanced gastric cancer include the chimeric anti-mesothelin antibody amatuximab and the antibody drug conjugate BAY94-9343. Amatuximab has been shown to kill mesothelin-expressing cell lines by antibody-dependent cell-mediated cytotoxicity and also inhibits the interaction between mesothelin and CA-125(28). BAY 94-9343 is composed of a fully human IgG1 anti-mesothelin monoclonal antibody that is linked to the maytansine derivative DM4 and it is currently undergoing phase I clinical investigation in a number of solid tumors.

Additional strategies that warrant investigation in advanced gastric cancer include exploiting mesothelin as a target for chimeric antigen receptor T-cell therapy(26) or utilizing the mesothelin tumor vaccine CRS-207(19). These drugs have mechanisms of action and toxicity profiles that are distinctly different and non-overlapping with standard approved chemotherapy regimens used in metastatic gastro-esophageal cancer.

Interestingly we did not see a significant difference in mesothelin expression between the histologically distinct subtypes of diffuse and intestinal type gastric cancer. Tumor location was also not a contributing factor. HER2 positive disease on the other hand is almost exclusively observed in intestinal type carcinomas and tumors at the gastro-esophageal junction and proximal stomach have higher expression(2). Patients with diffuse gastric cancer have therefore been limited to receiving cytotoxic chemotherapy alone. Unfortunately even patients with HER2 positive disease only derive limited benefits when trastuzumab is added to chemotherapy with a median overall survival of 13.8 months vs 11.1 months(2).

C-met has been proposed as a promising new target in advanced disease and a number of phase III trials are now in progress combining MET inhibitors with chemotherapy in advanced gastric cancer. In our study we found c-met positivity in 6.6% of carcinomas limiting the number of patients that could benefit from c-met targeted therapy. In a recent phase II study the anti-HGF monoclonal antibody rilotumumab was investigated combined with chemotherapy with the primary endpoint of progression free survival (PFS). PFS was 5.7 months in rilotumumab treatment arms vs 4.2 months in placebo group (HR 0.60, 80% CI 0.45-0.79; p=0.016) (29). Unfortunately a significant increase in overall survival was not reported although a phase III study is ongoing. In our study we identified that only 8 tumors out of 127 were c-met positive (c-met low 5% and c-met high 1%).

The Cancer Genome Atlas Research Network (TCGA) has recently comprehensively characterized gastric cancers and has proposed four subtypes (1) tumors positive for Epstein-Barr virus, which display recurrent PIK3CA mutations, extreme DNA hyper-methylation, and amplification of JAK2, PD-L1 and PD-L2; (2) microsatellite unstable tumors, which have rare mutations of HER2 and HER3, EGFR and PIK3CA; (3) genomically stable tumors, which are enriched for the diffuse histological variant and have

newly described mutations of RHOA or fusions involving RHO-family GTPase-activating proteins; and (1) tumors with chromosomal instability, which show marked aneuploidy and focal amplification of receptor tyrosine kinases such as amplification of VEGFA and frequent amplifications of cell cycle mediators (CCNE1, CCND1 and CDK6)(30). Here we report that in addition to these newly described targets that mesothelin is consistently and highly expressed across the continuum of upper GI carcinomas making it a potentially very promising target of interest. With approximately 60% of advanced gastric cancers demonstrating mesothelin expression and with a number of novel drugs undergoing early phase clinical investigation it is hoped that targeting this glycoprotein may improve patient outcomes in a tumor that has seen limited breakthroughs in recent decades.

Acknowledgments

Support: This research has been sponsored in part by the Sidney Kimmel Comprehensive Cancer Center Core Grant P30 CA006973 and by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research

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Figure 1.

Representative images of immunohistochemistry for mesothelin, E-cadherin, Her2/Neu and c-met.

Figure 2.

FACS analysis of mesothelin surface expression of AGS cells.

Live cells were labeled with anti-mesothelin antibody and then antibody was detected with PE-conjugated secondary antibody. A) Representative histogram of PE detection in greater than 20,000 AGS cells (shaded curve) compared to control (unfilled curve). B) WST-8 cell viability assay of SS1P treated AGS cells. 100% viability was normalized to measurement for untreated cells and 0% viability to cells treated with 10 mcg/mL cyclohexamide which produces complete cell death. A representative cytotoxicity curves is shown. Each point represents the average of triplicate measurements. The dotted horizontal line marks 50% cell killing. The vertical arrow indicates the IC₅₀ for the run. The average IC₅₀ after 8 experiments was 0.4 ng/mL.

Table 1

Characteristics of Patients/Tumors (N=127)

Characteristic	Number of Patients/Tumors (%)
Sex	
Male	86 (68%)
Female	41 (32%)
Age	
Median Age	63
Range	28-89
Tumor Location/Lauren Classification	
<i>Stomach</i> (N = 116)	
Intestinal type	61 (48%)
Diffuse type	48 (38%)
Mixed/unclassified	7 (5%)
<i>Gastro-esophageal junction</i> (N = 11)	11 (9%)
Tumor Stage (TNM)	
Stage 0 (Tis)	1 (1%)
Stage I (IA+IB)	23 (18%)
Stage II	31 (25%)
Stage III (IIIA+IIIB)	40 (31%)
Stage IV	32 (25%)
Mesothelin Status (N = 127)	
Negative (0 – 1+)	63 (50%)
Low positive 2+	30 (24%)
High positive 3+	34 (26%)
HER2-neu Status (N = 127)	
IHC negative (0 – 1+)	116 (91%)
IHC equivocal (2+)	2 (2%)
IHC positive (3+)	9 (7%)
E-Cadherin Status (N = 126)	
Negative (0-1+)	11 (9%)
Low positive 2+	39 (31%)
High positive 3+	76 (60%)
c-Met expression (N = 122)	
IHC negative	114 (93%)
Low positive	7 (6%)
High positive	1 (1%)

Table 2

Positive mesothelin staining using three different mouse monoclonal anti-mesothelin antibodies.

	5B2 (%)	D4038 (%)	2C6 (%)
Papillary serous carcinoma, ovary	31/33 (94%)	30/33 (91%)	33/33 (100%)
Adenocarcinoma, pancreas	31/35 (89%)	33/36 (92%)	37/37 (100%)
Malignant mesothelioma	18/24 (75%)	17/24 (71%)	21/23 (91%)

Table 3

Semi-quantitative scoring criteria for mesothelin and e-cadherin stains

Score	Staining
0	No staining
1 +	Weak staining only or Moderate staining in <10% of tumor cells
2+	Moderate to strong staining in >10% but less than 50% of tumor cells
3+	Moderate to strong staining >50% of tumor cells