# Could the immune response in the sentinel lymph nodes of gastric cancer patients be the key to tailored surgery?

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***Background.*** *Precise detection of downstream, nonsentinel lymph node metastases is the key to implementation of the sentinel lymph node concept in gastric cancer. To overcome the problem of complex lymphatic drainage, micrometastases, and skip metastases, we investigated the feasibility of tumor cell detection in sentinel lymph nodes, using flow cytometry as well as studied immune suppression in the sentinel lymph node as a potential marker of downstream lymph node metastases.*

***Methods.*** *In 21 patients with gastric cancer, the sentinel lymph nodes extracted during operation subjected to frozen sections and flow cytometry. The tumor cells were defined with the cell surface markers CEACAM and EpCAM. Simultaneously, the cell densities of different subsets of T cells were determined.*

***Results.*** *The sensitivity and specificity of the determination of nodal status with flow cytometry for tumor cell detection was 100% and 63%, respectively, as seen in frozen sections. Correlations with nonsentinel lymph node metastases were seen for $CD127^{low}CD25^{high}$ and $CD45^{neg}CD127^{low}CD25^{high}$ cell densities, relative proportion of $CD45RA^{neg}CD127^{low}CD25^{high}$ cells, frozen sections results, lymphangial invasion, and tumor size ($P \leq .043$ each). Multivariate analysis identified the relative proportions of $CD45RA^{neg}CD127^{low}CD25^{high}$ cells as the only significant predictor for downstream nonsentinel lymph node metastases ($P = .028$; 95% confidence interval, 1.107–5.780). The predictive value of combined detection of flow cytometry tumor cells and the relative proportion of $CD45RA^{neg}CD127^{low}CD25^{high}$ cells for nodal stage determination was 91%.*

***Conclusion.*** *Combined detection of tumor cells and $CD45RA^{neg}CD127^{low}CD25^{high}$ cells in sentinel lymph nodes with flow cytometry predicts accurately nonsentinel lymph node metastases. (Surgery 2016;160:613-22.)*

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Since its introduction in surgery for gastric cancer in 1992,[1] many attempts have been made to implement the concepts of sentinel lymph node (SLN) in routine gastric cancer operations. Although this concept has helped to better define melanoma and breast cancer and has introduced organ-sparing operations, it has not become standard practice in most other fields of surgery. In gastric cancer, the main obstacles are the complexity of lymphatic drainage, frequent micrometastases, and skip metastases.[2-6] Due to the multidirectionality of the lymphatic pathways, there is usually >1 SLN that drains a tumor. Furthermore, the first metastatic deposits in gastric cancer are of a small size, and LN metastases can develop even in patients who are node-negative on routine histology.[7,8] These LN metastases seem to develop from micrometastases that can be missed on routine, histologic LN examination.[7,8] Many studies have overcome this problem by employing more meticulous LN sectioning or immunohistochemical analysis and/or implementation of quantitative real-time polymerase chain reaction (RT-qPCR) for detection of micrometastases.[9,10] Although RT-qPCR is the most sensitive and specific method used to date,[10,11] the problem with extensive, step-sectioning of LNs and these molecular methods is that they are time-consuming and labor-intensive, preventing their intraoperative use in most centers.

Recently, flow cytometry is a useful method for diagnosis of hematologic malignancies and for

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malignant effusions of various epithelial neoplasms.[12,13] Flow cytometry has been shown to be a reliable and rapid method that combines the high specificity of morphologic methods and the high sensitivity of molecular methods,[12,13] and it is a potential counterpart to other more elaborate molecular methods. Regardless of the sensitivity of any method used, however, the problem of skip metastases remains unsolved. Therefore, another marker for downstream, nonSLN metastases would improve the accuracy, where skip metastases make SLN analysis alone less effective.

The SLNs appear to be one of the first draining sites of metastasing tumor cells as well as the first lymphoid organs to respond to tumor antigen stimulation.[14-20] Many studies have demonstrated immunosuppression in SLNs in patients with LN metastases.[19,20] The immunosuppression arising in the SLNs that first encounter the tumor antigens is seen in structural changes to dendritic cells,[18] a decrease in cytotoxic T cell density, and upregulation of $CD45RA^{neg}CD127^{low}CD25^{high}$ (activated) regulatory T cells (Tregs). Although these changes predict downstream, LN metastases in squamous cell cancer of the head and neck,[19,20] the value in gastric cancer is still under study. We introduced flow cytometry screening of SLNs for the presence of tumor cells, combined with an analysis of the immunologic changes in the SLNs that might define the presence of downstream, nonSLN metastases. Thus, we have investigated the role of flow cytometry in SLN tumor-cell detection as well as the density of $CD45RA^{neg}$ $CD127^{low}CD25^{high}$ Tregs in SLNs of patients with gastric cancer as potential predictive factors for nonSLN metastases.

## METHODS

**Patients.** The SLNs of 21 patients (13 men, 8 women; mean age ± standard deviation, 61 ± 12 years) with histologically verified gastric cancer were collected. The SLN mapping was performed as described elsewhere.[21-23] Briefly, Patent Blue V dye (2.5% Patent Blue V Sodium; Guerbet, Roissy, France) was injected intraoperatively in 4–5 sectors around the tumor. The drainage pattern was followed to the first draining LN. The SLN was extracted in 26 ± 8 minutes and halved through the largest diameter. Half of the specimen was subjected to frozen-section analysis, and the other half was put in phosphate-buffered saline and sent immediately to the flow cytometry laboratory for additional analysis. A D2 gastrectomy was then performed according to the third edition of the Japanese Gastric Cancer Association guidelines. The tumor, nodes, and metastases (TMN) stage was determined after the operation according to the classifications of the seventh Union for International Cancer Control. The histology reports confirmed the neoplasms as 1 carcinoma in situ, 1 T1a, 6 T1b, 8 T3, 4 T4a, and 1 T4b. Ten patients were node-negative (42%), with the remaining 11 patients node-positive (48%). The tumors were located in the distal third of the stomach in 10 patients (48%), in the middle third in 9 patients (43%), in the proximal third in 1, and at the esophago-gastric junction in 1. A subtotal distal gastrectomy was performed in 10 patients (48%), and a total gastrectomy in 11 (53%). All patients gave informed consent before being included in the study, which was approved by the National Ethnics Committee (University Clinical Centre Maribor, Slovenia; No. 153/02/09). The full patient characteristics are given in Table I.

**Intraoperative analysis of the SLN/frozen sections.** The operations proceeded as standard gastrectomies with D2 lymphadenectomies irrespective of the final frozen-section results. The SLN samples >5 mm in size were halved, and a 4-$\mu$m-thick section was examined from each half of the sample. In samples <5 mm, only 1 4-$\mu$m-thick section was examined from 1 level of the SLN (for the largest diameter). The sections were stained with hematoxylin and eosin. All of the samples were also paraffin embedded for additional sampling at different levels. In the final histologic examination of the SLNs, 2, 4-$\mu$m-thick sections were analyzed at 250-$\mu$m intervals until a metastasis was found or until the whole LN had been sectioned. The neighboring sections were stained with hematoxylin and eosin and immunohistochemically with anti-cytokeratin antibodies. Immunohistochemical staining was performed with an auto-immunostainer (Ventana Benchmark XT; Ventana Medical Systems, Inc, Tucson, AZ). The visualization process was performed with diaminobenzidine detection kits (Ventana UltraView Universal; Ventana Medical Systems) according to the manufacturer's protocol using a AE1/AE3 anti-cytokeratin antibody (1:100 dilution; Dako, Carpinteria, CA).

**Flow cytometry immunophenotyping.** The cells were suspended in 100 $\mu$l phosphate-buffered saline with 0.02% (w/v) Ethylenediaminetetraacetic acid (EDTA) and immunostained for 30 minutes with APC-Cy7–conjugated CD45 and pycroerythrin-conjugated CD326 (both from Beckton Dickinson, Biosciences, San Jose, CA). After red blood cell lysis with ammonium chloride, the cells were washed and stained with 7-amino-