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Can flow cytometry reinvent the sentinel lymph node concept in gastric cancer patients?

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ABSTRACT

Background: The focused sentinel lymph node (SLN) concept we proposed previously relied on real time-quantitative polymerase chain reaction (RT-qPCR) to detect tumor cells, which is too elaborate for intraoperative use. Therefore, we evaluated flow cytometry for intraoperative detection of tumor cells in SLNs.

Methods: Sixty-five consecutive gastric cancer patients were included. SLN analysis was carried out for a single SLN from each patient, using the molecular methods of RT-qPCR (first 30 patients) and flow cytometry (final 35 patients). All LNs underwent hematoxylin and eosin staining and immunohistochemical examination.

Results: Extraction of the SLN from a high-risk station was an important determinant for accurate prediction of LN metastases. For RT-qPCR, the sensitivity and specificity of detection were 72.7% and 81.8%, respectively, and for flow cytometry, 36.8% and 100%, respectively. When only high-risk SLNs were analyzed and specimens with <10% viability of leukocytes were excluded, the sensitivity and specificity of flow cytometry were 60% and 100%, respectively. Multivariate analysis identified significant predictors for LN metastases as the molecular method of SLN analysis ($P = 0.021$; 95% confidence interval [CI]: 1.304-24.284) and lymphovascular invasion ($P = 0.002$; 95% CI: 2.142-28.555). In subgroup analysis of high-risk SLNs, only RT-qPCR was a significant predictor for LN metastases ($P = 0.016$; 95% CI: 1.581-91.084).

Conclusions: Flow cytometry of high-risk SLNs, excluding specimens with low cell viability is a rapid, cost-effective, widely obtainable, and highly specific method for SLN metastases detection although it lacks the necessary sensitivity. Therefore, it cannot be recommended as a stand-alone method for the detection of LN metastases during operations.

Introduction

Since the first reported use of the sentinel lymph node (SLN) concept in gastric cancer patients by Miwa *et al.*,[1] there has been immense interest in applying this to clinical practice.[1-6] However, after more than 20 years of intensive research, no changes in the surgical treatment of gastric cancer patients have been indicated. As none of the methods defined to date

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can reliably determine LN metastases preoperatively and during operations, functional preservation of local wedge resections and SLN basin dissection remain elusive. A more precise method for LN metastases detection thus remains to be found. Until then, these patients still have to be treated with gastric resections and radical lymphadenectomies that are accompanied by many functional difficulties, such as dumping syndrome, malnutrition, intraabdominal adhesions, and others.[7]

Gastric cancer shows different properties to those with breast cancer and melanoma, where the concept of the SLN is a vital part of treatment. The stomach is known to have a wide drainage field due to the complex lymphatic draining pathways. Gastric cancer can often form metastases as isolated tumor cells that are difficult to detect in frozen sections or using routine histology. Both of these problems have been addressed through the use of molecular analysis of many LNs, with the consequent greatly improved accuracy. However, unfortunately, both of these problems have made these methods more impractical, as they are time consuming, costly, and are not widely available.[1-6] We therefore proposed the concept of focused SLN analysis.[8-10] We have shown that analysis of only one or two high-risk SLNs with a highly sensitive method can produce reliable results in a shorter time.[8-10] However, as our method relies on real time-quantitative polymerase chain reaction (RT-qPCR), this concept is still restricted in its use to selected hospitals.

Flow cytometry has been traditionally used for analysis of liquid tissues, such as in the diagnostics of hematological malignancies. It can be used to screen millions of cells and has sensitivity close to that of molecular methods.[11,12] This has made flow cytometry an attractive alternative to immunohistochemistry and RT-qPCR. However, this requires that the LN tissue is provided as a single-cell suspension.

In the present study, we evaluated the use of flow cytometry for the detection of tumor cells in SLNs and to correctly stage gastric cancer patients during operations. As flow cytometry is a rapid and more widely available method than RT-qPCR, it should make our focused SLN concept even more applicable for intraoperative use. We therefore analyzed the accuracy of flow cytometry in the intraoperative prediction of LN metastases and compared this with RT-qPCR.

Methods

Patients

Sixty-five consecutive gastric cancer patients (42 men, 23 women; mean age, 64 ± 12 years) participated in this study. The study was approved by the National Ethnics Committee (University Clinical Centre Maribor, Slovenia; No. 153/02/09), and all of these patients gave their informed consent before inclusion.

Sentinel lymph node mapping and extraction

The LN preparation and SLN mapping and extraction were performed as described previously.[8-10] SLN navigation was performed using Patent Blue V dye (2.5% Patent Blue V Sodium; Guerbet, Roissy, France), which was injected intraoperatively in 4-5 sectors around the tumor (Fig. 1).[8-10] The first draining LN was extracted in a mean time of 21 ± 9 min and was halved through its largest diameter. One half of the SLN of all the patients was sent for conventional histology, with frozen sections prepared, and hematoxylin and eosin staining and immunohistochemical examinations were carried out. The other half of the SLN was analyzed with either RT-qPCR, in the first group of consecutive patients (n = 30; RT-qPCR group), or flow cytometry, in the second group of consecutive patients (n = 35; flow cytometry group). For the RT-qPCR, the half of the SLN was immediately placed in RNA Stabilization Reagent (RNAlater; Qiagen, Hilden, Germany). These submerged specimens were kept in this reagent at 0°C-4°C and were analyzed within a month.[8] For the flow cytometry group, the half of the SLN was placed in phosphate-buffered saline and was immediately sent to the flow cytometry laboratory for further analysis.[10]

Tumor surgery and analysis

A D2 gastrectomy was performed for all patients, according to the third Japanese Gastric Cancer Association guidelines.[13] The tumor, node, metastases stage was determined after each operation, according to the seventh Union for International Cancer Control classification.[13] The histology reports confirmed the tumors as the following: carcinoma *in situ*, 1 (1.5%); T1a, 12 (18.5%); T1b, 13 (20.0%); T2, 10 (15.4%); T3, 23 (35.4%); T4a, 5 (7.7%); and T4b, 1 (1.5%). Twenty-nine patients (44.6%) were node negative, whereas the remaining 36 (55.4%) were node positive. The tumors were predominantly located in the middle and distal third (middle, 29 [44.6%] and distal, 28 [43.1%]). Only five tumors (7.7%) were located at the gastroesophageal junction and three (4.6%) in the upper third. Most of the tumors showed poor differentiation (64.6%). The most common macroscopic appearance in early gastric cancer was ulcerated Murakami type III (10.8%) and in advanced gastric cancer, exulcerated Bormann type II (38.5%). Subtotal distal gastrectomy was performed in 26 patients (40.0%), total gastrectomy in 38 patients (58.5%), and proximal subtotal gastrectomy in 1 patient (1.5%). In each case, D2 lymphadenectomy was performed, with the median number of extracted LNs of 20 ± 12 (range; 10-75).

Intraoperative analysis of sentinel lymph node frozen sections

The frozen sections were prepared as described previously.[8-10] Neighboring sections were stained with hematoxylin and eosin, with immunohistochemical staining with anti-cytokeratin antibodies.[8-10,14] This immunohistochemical staining was performed with an autoimmunostainer (BenchMark XT; Ventana Medical Systems, Inc, Tucson, AZ). Visualization was performed using the ultraView Universal DAB Detection Kit (Ventana Medical Systems, Inc), according to the manufacturer's instructions.[8-10] For the detection of metastases, the Anti-Cytokeratin AE1/AE3 Antibody was used (1:100 dilution; Dako, Carpinteria, CA).