Histotype-based prognostic classification of gastric cancer

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Abstract

AIM: To test the efficiency of a recently proposed histotype-based grading system in a consecutive series of gastric cancers.

METHODS: Two hundred advanced gastric cancers operated upon in 1980-1987 and followed for a median 159 mo were investigated on hematoxylin-eosin-stained sections to identify low-grade [muconodular, well differentiated tubular, diffuse desmoplastic and high lymphoid response (HLR)], high-grade (anaplastic and mucinous invasive) and intermediate-grade (ordinary cohesive, diffuse and mucinous) cancers, in parallel with a previously investigated series of 292 cases. In addition, immunohistochemical analyses for CD8, CD11 and HLA-DR antigens, pancytokeratin and podoplanin, as well as immunohistochemical and molecular tests for microsatellite DNA instability and in situ hybridization for the Epstein-Barr virus (EBV) EBER1 gene were performed. Patient survival was assessed with death rates per 100 person-years and with Kaplan-Meier or Cox model estimates.

RESULTS: Collectively, the four low-grade histotypes accounted for 22% and the two high-grade histotypes for 7% of the consecutive cancers investigated, while the remaining 71% of cases were intermediate-grade cancers, with highly significant, stage-independent, survival differences among the three tumor grades (P = 0.004 for grade 1 vs 2 and P = 0.0019 for grade 2 vs grade 3), thus confirming the results in the original series. A combined analysis of 492 cases showed an improved prognostic value of histotype-based grading compared with the Lauren classification. In addition, it allowed better characterization of rare histotypes, particularly the three subsets of prognostically different mucinous neoplasms, of which 10 ordinary mucinous cancers showed stage-inclusive survival worse than that of 20 muconodular (P = 0.037) and better than that of 21 high-grade (P < 0.001) cases. Tumors with high-level microsatellite DNA instability (MSI-H) or EBV infection, together with a third subset negative for both conditions, formed the T8 cell-rich HLR group, the largest group among low-grade histotypes. Coexisting HLR proved to be a factor in improved prognosis in tumors with microsatellite instability (P = 0.0015 vs HLR/-MSI-H tumors) or DR type human leukocyte antigen expression (P = 0.033 vs HLR/HLA-DR+ tumors).

CONCLUSION: Identification of low- and high-grade histotypes can improve the prognostic assessment of a substantial proportion of gastric cancers in routine diagnostic practice.

Key words: Gastric cancer; High-grade histotype; Low-grade histotype; Lymphoid response; Epstein-Barr virus; Microsatellite instability

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INTRODUCTION

The difficulty of assessing the prognosis of gastric cancer using histological methods is well known and this is also reflected in the essentially descriptive character of presently used classifications[1-4]. However, several histotypes characterized by lower malignant potential have been identified and separated from more common cohesive (so-called “intestinal”) or diffuse tumors. They include lymphocyte-rich cancer[5-7], mucinous nodular cancer[8], very-well-differentiated tubular cancer with an intestinal[9] or gastric[10] phenotype, and a low-grade subtype of diffuse desmoplastic cancer[11]. Similarly, various kinds of cancer with poor outcome have been identified, from poorly differentiated neuroendocrine carcinoma, small to large cell[12,13], to anaplastic diffuse cancer[13] or hepatoid[14], choriocarcinomatous[15] and adenosquamous carcinoma[16]. In addition, comparative genomic hybridization analysis has shown a clear relationship between the number and severity of genomic alterations and tumor histotype and prognosis[17,18].

The different behavior of these histotypes offered an opportunity to develop a three-grade system of prognostic evaluation, which, when applied to a large tumor series, was highly predictive of patient outcome[19]. However, the tumor series used in that study was substantially selected (1) to be representative of all main stages (intramucosal cases apart) and histological types of the disease; and (2) to include uncommon histological subtypes or variants, as well as earlier invasive stages (submucosal or confined to muscularis propria). Therefore, in order to ascertain the effectiveness of the system in routine diagnostic work, a continuous, homogeneous series of advanced cancers needs to be evaluated.

In this study, we retrospectively identified prognostic histotypes according to previously reported criteria[19] in a consecutive series of advanced (muscularis propria invasion or beyond) gastric cancers collected at Varese General Hospital during 1980-1987, and we tested such histotypes as potential predictors of patient outcome and compared the results with those of the original Pavia series. During the study, we realized that we needed to investigate further mucinous and lymphocyte-rich cancers, due to discrepancies concerning: (1) the impact of histology or stage on mucinous cancer prognosis[20-23], and (2) the contribution of tumor microsatellite instability, Epstein-Barr virus (EBV) infection and DR type human leukocyte antigen (HLA-DR) expression, rather than lymphoid cell response per se, to the natural history of lymphocyte-rich neoplasms[24-27]. Therefore, mucinous and lymphocyte-rich tumors from both series were combined to obtain tumor groups large enough to allow appropriate investigation.

MATERIALS AND METHODS

Tissue samples
A consecutive series of 200 invasive (T2-T4) gastric cancers were retrieved from the files of the Anatomic Pathology Service, for patients who had undergone potentially curative surgery at Varese General Hospital during 1980-1987. The clinicopathological and follow-up data of all the patients were carefully collected from hospital records, interviews with family doctors, and the Varese Province Tumor Registry. One hundred and eighty-five of the cases had already been the subject of a previous investigation[19]. Eight cases were operated on during January 1980-April 1987 and were not considered in the previous study because the available clinical or follow-up documentation was incomplete. This information was retracted, appropriately documented and added to the present study to ensure the continuous pattern of the patient series, together with seven more cases operated on in May-June 1987. The original tumor node metastasis (TNM) stage assessment of each tumor was revised according to the criteria of the 2002, 6th Edition American Joint Committee on Cancer system[28]. No antibiotic therapy had been given to the patients. For survivors, the follow-up period was prolonged until 2008; a median follow-up of 159 mo was recorded.

Immunohistochemical analysis
Archival and newly cut paraffin sections were stained with hematoxylin-eosin, Alcian blue-periodic acid Schiff or the immunoperoxidase procedure using antibodies directed against h-MLH1 (G-168.15 clone; Pharmingen, San Diego, CA, United States), hMSH2 (Fe11 clone; Oncogene, Cambridge, MA, United States), hPMS2 (clone A16-4; Oncogene, CA, United States), CD11c antigen (C8/144B clone; Dako, Glostrup, Denmark), CD11e antigen (SD11 clone; Novac opportum Laboratories, Newcastle, United Kingdom), pancytokeratin (AE1/AE3 clone; Novac opportum Laboratories), HLA-DR (LN3 clone; Biotest, Dreieich, Germany) and podoplanin (D2-40 clone; Biocare Medical, Concord, CA, United States) as previously reported[29,30].

Molecular analysis
In situ hybridization for the EBER1 gene of EBV was performed as described previously[13,14]. Microsatellite instability was assessed at Bat 25, Bat 26, BAT40, DSS346 and D2S123 loci. Tumors with instability involving at
least two of the five loci were classified as highly instable (MSI-H), while those with only one instable locus were classified as low instable (MSI-L) and included in the MSI negative tumor group together with microsatellite stable cases.[2,19,30].

**Morphological analysis**

Tumor histotypes were identified as described previously.[8-11,19]. In particular, very-well-differentiated tubular (VWDt) cancer is characterized by glands with moderately atypical, polarized cells arranged in a monosatratified epithelium, low-grade diffuse desmoplastic cancer shows fibroblast-rich desmoplasia surrounding individual (or minute groups of) moderately atypical tumor cells, while mucinous nodular forms extracellular mucin lakes with expanse borders in which isolated signet ring cells or cords of mucin-producing tumor cells are freely floating.

To increase the diagnostic accuracy of lymphocyte-rich tumors, as well as intratumor CD8 T cell counts, intraepithelial T8 cells (i.e., cells infiltrating tumor aggregates so as to contact neoplastic cells directly, with the exclusion of purely stromal T8 cells) were also counted[7,31], and an evaluation of dendritic cells was added[27]. Thus, in this study, classification of a lymphocyte-rich tumor as high lymphoid response (HLR) required one of the following: (1) a lymphoepithelial type histological pattern with an overwhelming lymphoepithyte infiltrate dissecting tumor cells; or (2) > 400 intratumor and/or >200 intraepithelial CD8-positive cells in 10 high-power fields (HPFs), coupled with a band of lymphoid cells rich in CD8 T cells and CD11c dendritic cells surrounding expanse tumor nodules.

Anaplastic cancers were characterized by small to large, cytokeratin-positive cells with highly atypical nuclei, with or without prominent nucleoli and with or without signs of poor neuroendocrine differentiation, high cellularity, scarce stroma, and high proliferative rates (> 20 mitoses/10 HPFs)[11,12]. During characterization of the mucinous infiltrative tumors, it was found that those showing local infiltration of peritumoral tissues in the absence of prominent lymphoivasion or angioinvasion had a less severe prognosis. Therefore, in this study, infiltrative tumors lacking vascular invasion or with only sporadic lymphoivasion were added to the grade 2 group, together with ordinary cohesive and diffuse cancers, while only predominantly lymphoivasive (two or more foci per microscopic tumor sections) or angioinvasive cases remained in the grade 3 group together with anaplastic cancers, as in the original classification.[19]. Cases showing a coexistence of two or more histological patterns were classified according to their prevalent histotype, provided that all the components were low-grade; otherwise, they were classified according to their higher grade component.

A reproducibility test involving two senior pathologists (Solcia E and Capella C) gave a κ value of 0.84 concerning interobserver agreement for five main histotypes (cohesive, diffuse, mucinous, anaplastic and HLR), a κ of 0.81 agreement for nine subtypes (HLR, VWDt, ordinary cohesive, low-grade diffuse desmoplastic, ordinary diffuse, mucinous, anaplastic and HLR), and a κ of 0.79 agreement for the three histotype-based grades (low, intermediate and high).

| Stage | n (%) | Death rate | 95% CI | Cox survival analysis |
|-------|-------|------------|-------|-----------------------|
| I     | 40 (20)| 1.63       | 0.81-3.25 | 1                     |
| II    | 61 (30.5) | 13.67     | 10.06-18.56 | 5.64 | 2.63-12.09 | < 0.001* |
| III   | 68 (34) | 26.04     | 19.74-34.46 | 9.49 | 4.45-20.24 | < 0.001* |
| IV    | 31 (15.5) | 52.66       | 36.11-76.79 | 18.90 | 8.41-42.46 | < 0.001* |

*P = 0.001 vs IV; **P = 0.015 vs II; ***P = 0.004 vs III. CI: Confidence intervals; HR: Hazard ratio.

From the previously investigated Pavia series of 294 cases[19], 292 cases (two tumors had to be excluded because there was no remaining tumor tissue) were considered for comparative analysis with the Varese series, as well as for a joint reinvestigation of both series looking at mucinous and HLR tumors according to the above criteria. In addition, the prognostic value of the histotype-based grading system was compared with that of the commonly used Lauren classification[10].

**Statistical analysis**

Statistical analysis was performed using Stata version 11 (Stata Corporation, College Station, TX, United States). All tests were two-sided. Categorical variables were described with counts and percentages and compared with the Fisher exact test. Continuous variables were described with median and quartiles, and compared with the Kruskal-Wallis test. Death rates per 100 person-years, with 95% confidence intervals (CIs), and Kaplan-Meier estimates were computed to describe survival. The Cox model was used to assess the prognostic role of the considered variables; both univariate and bivariate models (inclusive of stage) were fitted. The hazard ratio (HR) and 95% CI were reported. Proportional hazard assumptions were satisfied in all cases. The Harrell’s c statistic was computed to assess model performance (discrimination ability); a value of 0.5 indicating no discrimination and a value of 1 indicating perfect discrimination.

**RESULTS**

**Characterization of the Varese continuous series**

The Varese consecutive, non-selected series of 200 advanced (T stages or above) gastric cancers is described in Table 1 according to stage and patient survival. Compared with the original Pavia selected series, which also included a substantial number (44 cases) of deeply submucosal (penetrating T1b) tumors, the present series showed more advanced tumors (Stage III + IV: 49.5% vs 39% of the original series; Stage II: 30.5% vs 25%). A clear step-wise, stage-dependent behavior emerges from the survival analysis.

Of the 200 tumors, 44 (22%) had low-grade, 14 (7%) high-grade and 142 (71%) intermediate-grade histotypes.
Survival analysis according to histotype-based grade is outlined in Table 2 and Figure 3. The more favorable behavior of grade 1 compared to grade 2 and of grade 2 compared to grade 3 tumors is evident.

In Table 2, univariate analysis of the Varese series after reclassification according to Lauren\cite{1} shows a significantly worse prognosis for the diffuse compared to intestinal and unclassified types [model: $\chi^2 (2) = 8.67$, $P = 0.013$]. However no significant difference was observed among the same cases in the stage-inclusive bivariate analysis (model: $P = 0.341$ for Lauren classification), while no difference was found by either univariate (model: $\chi^2 (2) = 4.87$, $P = 0.087$) or bivariate (model: $P = 0.342$) analysis among the 292 tumors in the Pavia series. In both the Varese and Pavia series, the Harrell’s concordance ($c$) test showed a higher efficiency of the histotype-based grading ($c = 0.63$ and $0.73$, respectively) compared to the Lauren classification ($c = 0.57$ and $0.55$).

Individual histotypes in the three grades are detailed in Table 3, first column. It appears that, while ordinary cohesive or diffuse and HLR tumors form a substantial group, the number of other histotypes is too low to allow appropriate statistical analysis.

**Joint analysis of 492 cases from both Varese and Pavia series**

When corresponding tumors of the two series were analyzed jointly (Table 3), the more favorable behavior of grade 1 compared to grade 2 tumors and of the latter compared to grade 3 cases was confirmed. In addition,
the resulting number of the rare histotypes was sufficient to allow survival analysis of each histotype. Thus, the prognostic similarity of types belonging to the same grade and their significant difference from those of other grades was assessed.

Reinvestigation of mucinous and high lymphoid response tumors

It also appears from Table 3 that mucinous neoplasms, when appropriately reclassified as muconodular, ordinary mucinous and highly invasive mucinous cancers, may form three prognostically different histological subsets, as confirmed by separate Cox univariate and stage-inclusive

Figure 2  Muconodular, well-differentiated tubular, diffuse desmoplastic and high lymphoid response. A: Low-grade muconodular cancer with expansile growth (hematoxylin-eosin, × 20); in the inset, note tumor cells floating inside mucin, free of contact with stroma (× 400); B: intermediate-grade, locally infiltrative mucinous cancer (hematoxylin-eosin, × 200); C: massive lymphoinvasion of a high-grade mucinous cancer (podoplanin immunoperoxidase-hematoxylin, × 200); D: Diffuse anaplastic cancer invading the muscularis propria (hematoxylin-eosin, × 200).

Figure 3  Kaplan-Meier survival estimate of 200 gastric cancers according to histotype-based grade.

Figure 4  Cox survival analysis of 200 gastric cancers according to histotype-based grade and according to Lauren classification.

Table 2  Survival analysis of 200 gastric cancers according to histotype-based grade and according to Lauren classification

| Grade | n (%) | Death rate | 95% CI | HR | 95% CI | P value |
|-------|-------|------------|--------|----|--------|---------|
| 1     | 44 (22) | 3.35 | 2.02-5.55 | 1 |
| 2     | 142 (71) | 17.24 | 14.16-21.90 | 3.5 | 2.02-6.05 | < 0.001 |
| 3     | 14 (7) | 95.66 | 54.33-168.45 | 9.64 | 4.41-21.06 | < 0.001 |
| Lauren type | | | | | | |
| Intestinal | 116 (58) | 8.86 | 6.93-11.31 | 1 |
| Diffuse | 50 (25) | 21.08 | 15.20-29.22 | 1.71 | 1.13-2.57 | 0.011 |
| Unclassified | 34 (17) | 18.4 | 12.53-27.03 | 1.7 | 1.07-2.68 | 0.023 |

P = 0.001 vs grade 2; P = 0.019 vs grade 2. Harrell’s c = 0.63. 1: Univariate model: \( \chi^2 \) (2) = 8.67, P = 0.013. Harrell’s c = 0.57. Stage-inclusive model: P = 0.341 for Lauren classification. CI: Confidence intervals; HR: Hazard ratio.
Table 3  Survival analysis of 492 tumors (Varese + Pavia series) according to histotype and grade

|               | n (%)         | Cox survival analysis                                      |
|---------------|---------------|------------------------------------------------------------|
|               | Varese series | Joint series      | Death rate | 95% CI    | HR1 | 95% CI | Univariate | With stage |
| Grade 1       | 44 (22)       | 132 (26.8)       | 2.54       | 1.80-3.57 | 1   |         |            |            |
| HLR           | 38 (19)       | 82 (16.6)        | 3.67       | 2.50-5.39 | 1   |         |            |            |
| WD tubular    | 2 (1)         | 13 (2.6)         | 0          |           |     |         |            |            |
| Lg diff.desm  | 2 (1)         | 17 (3.5)         | 1.50       | 0.48-4.64 | 0.43| 0.13-1.42| 0.167      | 0.109      |
| mucinous      | 2 (1)         | 20 (4.1)         | 1.67       | 0.63-4.44 | 0.51| 0.18-1.46| 0.211      | 0.271      |
| Grade 2       | 142 (77)      | 307 (62.4)       | 15.97      | 13.96-18.28| 4.91| 3.40-7.11|< 0.001 |< 0.001     |
| Cohesive ord. | 4 (2)         | 10 (2.0)         | 11.50      | 5.16-25.59| 2.16| 0.89-5.24| 0.090a     | 0.962c     |
| Diffuse ord.  | 43 (21.5)     | 101 (20.6)       | 18.09      | 14.36-22.79| 3.96| 2.53-6.22|< 0.001 |< 0.001     |
| Grade 3       | 14 (7)        | 53 (10.8)        | 108.84     | 82.02-144.43| 18.47| 11.56-29.50|< 0.001 |< 0.001     |
| Mucinous Hg.  | 3 (1.5)       | 21 (4.3)         | 100.0      | 64.07-160.0| 11.54| 6.32-21.48|< 0.001 |< 0.001     |
| Anaplastic    | 11 (5.5)      | 32 (6.5)         | 120.0      | 80.01-170.0| 14.11| 8.17-24.39|< 0.001 |< 0.001     |

1For grades, based on grade 1; for histotypes, based on high lymphoid response (HLR) type. 2P = 0.005 vs mucinous and P < 0.001 vs mucinous. 3P = 0.037 vs mucinous and P < 0.001 vs mucinous. 4P < 0.001 vs grade 2. Harrell’s c = 0.69 for the 3 grades and 0.71 for the 9 histotypes. CI: Confidence intervals; HR: Hazard ratio; WD: Well differentiated; diff.desm: Diffuse desmoplastic; ord.: Ordinary; Hg.: High-grade.

Table 4 Three subtypes of high lymphoid response tumors and comparison with non-high lymphoid response high-level microsatellite DNA instability cases (Pavia and Varese series)

|               | n (%) | Death rate | 95% CI | Cox survival analysis |
|---------------|-------|------------|--------|-----------------------|
|               |       |            | HR     | 95% CI | Univariate | With stage |
| HLR           |       |            |        |        |            |            |
| EBV           | 24 (29.6) | 7.38 | 4.09-13.33 | 2.39 | 1.03-5.53 | 0.042 | 0.817 |
| MSI-H         | 40 (49.4) | 3.01 | 1.67-5.44 | 1     |            |        |        |
| HLA-DR        | 17 (21.0) | 2.27 | 0.85-6.06 | 0.81 | 0.26-2.53 | 0.711 | 0.393 |
| HLR           | 38 (48.7) | 12.29 | 8.24-18.33 | 3.64 | 1.78-7.47 |< 0.001 | 0.015 |

1One EBV’/MSI case omitted; 2% of all MSI-H cases, HLR’ or ’. HLR: High lymphoid response; EBV: Epstein-Barr virus; MSI-H: High-level microsatellite DNA instability.

Survival analyses, where ordinary mucinous cancers proved significantly worse than mucinous and better than highly invasive cancers (Table 3). Significant differences were also found between the three groups in terms of TNM stage, T level invasion and lymph node involvement (for all: P < 0.001, Fisher’s exact test) and even diameter (P < 0.001, Kruskal-Wallis test). In contrast, no significant trend (Cox univariate P = 0.126 and stage-inclusive P = 0.102) for better survival was noted among mucinous cancers as a whole; for those with cohesive vs diffuse or mixed histological patterns.

No survival difference was found between HLR and the three other low-grade histotypes or, among the HLR cases, between MSI-H and the EBV/MSI subset, while a trend for worse behavior of the EBV’ compared to the other subsets was noted by univariate analysis, which disappeared with stage-inclusive bivariate analysis (Table 4). EBV’ tumors also differed from the other two HLR subsets in showing significantly higher proportions of lymphoepithelioid histology (17/24, 71%, n = 5, 5/7, 9%, P < 0.001, Fisher’s exact test) and median intratumor T8 (107.5 µg 58.5 per HPF, P < 0.001, Kruskal-Wallis test). In contrast, 40 HLR MSI-H cases obviously showed more favorable behavior than their 38 non-HLR MSI-H counterparts (22 cohesive, 10 mucinous, three diffuse and anaplastic cancers), of which six were low-, 24 intermediate- and eight high-grade.

The proportion of mucinous neoplasms showing MSI-H (10/51; 19.6%) did not differ significantly from that of the whole tumor population (78/492; 15.9%) while remaining significantly lower than that of HLR cases (41/81; 50.6%). Notably, the 10 MSI-H cases were equally distributed among the three grades of mucinous cancers, being grade 1 [14/20 (20%)], grade 2 [1/10 (10%)], and grade 3 [5/21 (23.8%)].

For the combined analysis of HLR and HLA-DR status, 77 of the total 82 HLR tumors from both series had sufficient histological material left to allow reinvestigation, together with 202 randomly selected non-HLR tumors representative of all histotypes and stages. HLA-DR positivity in > 10% of tumor cells was found in 100/279 (35.8%) cases. Positive tumors showed a trend for lower death rate (5.97, 4.39-8.19 vs 9.41, 7.5-11.44) and improved survival (HR: 0.63, 0.44-0.91, P = 0.014) compared with HLA-DR cases, a behavior probably accounted for by the HLR’/HLA-DR’ subset, in which...
Potential implications for appropriate therapy

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coexistence of two or more distinct histological components within the same tumor, and by different host responses in terms of stromal response. A detailed histopathological classification should enable us to identify tumor subtypes that could provide useful prognostic and therapeutic information. The efficacy of a recently proposed classification system in predicting survival needs to be tested in a non-selected series of gastric carcinomas.

**Research frontiers**

This new histological classification takes advantage of the cytological, biological and architectural features of tumor cells to identify histological types (histotypes) of tumors with low, intermediate or high malignancy. Lymphoid and stromal reactions, which seem to play an important role in contrasting or favoring tumor growth, and consequently, resulting in a better or worse prognosis, also need to be taken into consideration.

**Innovation and breakthroughs**

The proposed three-grade system proved to be highly predictive of patient outcome. It identified low-grade (mucinous, well-differentiated tubular, diffuse desmoplastic and high lymphoid response), intermediate-grade (ordinary cohesive, diffuse and mucinous) and high-grade (anaplastic and mucinous invasive) gastric cancers, with highly significant stage-independent survival differences and had a better prognostic value compared to the Lauren classification.

**Application**

A careful histological examination of gastric cancers with the criteria proposed by the histotype-based prognostic classification was shown to be an effective tool in everyday diagnostic practice. Additional studies are necessary to identify histological and molecular parameters that could better characterize the large population of intermediate-grade cancers.

**Peer review**

The manuscript fits well with the scope of the journal, and is well written. It addresses a relevant aspect of gastric cancer, the exact histopathological assessment of the tumor and correlation of these data to clinical and molecular alterations.

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