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Three Molecular Subtypes of Gastric Adenocarcinoma Have Distinct Histochemical Features Reflecting Epstein-Barr Virus Infection Status and Neuroendocrine Differentiation

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Abstract

Current histopathologic classification schemes for gastric adenocarcinoma have limited clinical utility and are difficult to apply due to tumor heterogeneity. Elucidation of molecular subtypes of gastric cancer may contribute to our understanding of gastric cancer biology and to the development of new molecular markers that may lead to improved diagnosis, therapy, or

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prognosis. We previously demonstrated that Epstein-Barr virus infected gastric cancers have a distinct human gene expression profile compared to uninfected cancers. We now examine the histopathologic features characterizing infected (n=14) and uninfected (n=89) cancers, the latter of which are now further divided into two major molecular subtypes based on expression patterns of 93 RNAs. One uninfected gastric cancer subtype was distinguished by upregulation of three genes with neuroendocrine function (*CHGA*, *GAST*, and *REG4* encoding chromogranin, gastrin and the secreted peptide REG4 involved in epithelial cell regeneration), implicating hormonal factors in the pathogenesis of a major class of gastric adenocarcinomas. Evidence of neuroendocrine differentiation (molecular, immunohistochemical, or morphologic) was mutually exclusive of EBV infection. EBV infected tumors tended to have solid-type morphology with lymphoid stroma. This study reveals novel molecular subtypes of gastric cancer and their associated morphologies that demonstrate divergent neuroendocrine features.

Background

Gastric adenocarcinoma has remarkable morphologically heterogeneity, and many descriptive histologic classification schemes have been proposed over the past several decades[1,2,3,4,5,6]. Although some histologic types have prognostic and epidemiologic correlates, further progress is needed to classify tumors in a manner that impacts patient management. Variable clinical behavior and response to intervention attest to underlying biologic diversity. The lack of strong predictors of clinical outcome with any single histologic classification scheme highlights the complicated landscape of gastric cancer morphology which hampers comparative analysis across studies and underscores the need to devise novel methods of predicting response to current and future therapies.

The widely used Lauren classification divides the majority of gastric cancers into intestinal and diffuse types, and this dichotomous scheme has some clinicopathologic and epidemiologic relevance. However, there are other histologic patterns of prognostic significance, such as mixed[7,8,9,10], solid[7,8,11,12], and hepatoid[13,14], that are not distinguished under the Lauren scheme. Intratumoral heterogeneity is common, and different histologic features often coexist within a single tumor, thus confounding the morphologic classification attempts and raising concerns about their accuracy and reproducibility[7,15,16,17].

Gastric adenocarcinomas also have diverse pathogenesis, with *Helicobacter pylori* and Epstein-Barr virus (EBV) both designated as associated class 1 oncogenic pathogens by the World Health Organization (WHO). Approximately 10% of gastric adenocarcinomas harbor EBV infection in the neoplastic epithelial cells[18]. The prevalence of EBV infection is significantly higher (>80%) in the specific histologic type variably termed “gastric carcinoma with lymphoid stroma” or “lymphoepithelioma-like carcinoma”[19,20,21,22,23,24]. This tumor type is characterized by undifferentiated epithelial cells and an abundant lymphocytic infiltrate distributed uniformly throughout the tumor, similar to EBV-associated nasopharyngeal carcinoma. Gastric carcinoma with lymphoid stroma has a survival advantage over other histologic types[22,23], however the literature varies on whether EBV positivity alone or histopathologic appearance trumps in assigning prognosis [23,25,26,27,28]. When EBV-associated gastric cancers are classified according to their histologic patterns, some studies report predominance of Lauren intestinal type[26],

tubular type[26,29,30], Lauren diffuse type[20,23,27], and medullary/solid type[30,31]. This variability may illustrate the interpretative bias inherent in applying morphologic criteria.

High grade neuroendocrine carcinoma is another heterogeneous group of gastric malignancies classified and staged separately from gastric adenocarcinomas by the WHO[32]. It is frequently under-recognized because of the broad morphologic spectrum (from classic small cell carcinoma to poorly defined large cell carcinoma) and overlap with poorly differentiated adenocarcinoma[33,34,35,36]. Admixed adenocarcinoma components can be found in tumors of any of the neuroendocrine carcinoma subtypes, presenting a challenge in terms of the overall tumor classification. In fact, evidence of focal neuroendocrine differentiation has been found in over 50% of tumors otherwise classified as adenocarcinoma[37,38,39,40]. It is particularly common in signet ring cell carcinomas, leading some to propose that signet ring cells derive from a pluripotent neuroendocrine stem cell [41,42,43,44]. Molecular and infectious correlates of neuroendocrine differentiation have not been systematically investigated.

Molecular profiling holds promise for adding value in categorizing disease in a manner that is clinically meaningful. We previously demonstrated that EBV-infected gastric cancers have a distinct human gene expression profile compared to uninfected gastric cancers[45]. In the current study, we examine RNA expression patterns to further divide uninfected cancers into two principal molecular subtypes, and we evaluate associations with tumor histology and neuroendocrine gene expression. This study is the first, to our knowledge, to investigate the relationship between EBV infection and neuroendocrine differentiation in gastric cancer.

Materials and Methods

Tissue and molecular profiling

Consecutive gastric adenocarcinoma cases on which sufficient residual formalin-fixed paraffin-embedded tissue was available were assembled from pathology archives of three hospitals in disparate parts of the world, 30 from the University of North Carolina Hospitals in Chapel Hill, USA, 133 from Western Regional Hospital in Santa de Rosa, Honduras, and 24 from Wakayama Medical University, Wakayama, Japan. RNA profile data on this case cohort were previously published, and histopathology correlates are the subject of the current study. Studies were performed with approval of the University of North Carolina Biomedical Institutional Review board[45].

Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) and reviewed to identify tumor for subsequent macrodissection from unstained adjacent sections. Nucleic acid was extracted and RNA expression analysis was performed on the nCounter system (Nanostring) as previously described[45]. A custom panel of 96 RNAs (Gastrogenus v1™ panel) was measured which includes 73 human mRNAs of which 4 were housekeeper transcripts, 20 viral RNAs (7 latent and 9 lytic EBV mRNA transcripts, *EBER1* and *EBER2* non-coding RNAs, and two cytomegalovirus mRNAs), and 3 spiked ERCC RNA controls. The human RNAs were selected based on their proposed roles in gastric cancer pathogenesis, inflammatory response, and/or gastric cancer therapeutic targeting and monitoring. The viral RNAs were selected to detect EBV and to measure expression of

latent and lytic viral transcripts. After excluding samples with insufficient RNA or insufficient histopathologic material for morphologic assessment, a total 103 gastric carcinomas were included in the study. Of note, biopsies tended to yield better quality RNA than did resection specimens, possibly because biopsies tend to be immersed in formalin preservative much more quickly than are resected tumors.

Histologic analysis

All tumors were classified by both the Lauren system[1] and by the Carneiro system[7,46]. In the Carneiro classification of gastric cancer, mixed carcinomas consisting of both glands and infiltrating single cells, as well as solid carcinomas, are classified separately from purely gland-forming tumors and isolated-cell tumors. The latter two tumor categories correspond to Lauren intestinal and diffuse types, respectively. Solid carcinomas are composed of tumor cell sheets with little or no gland formation and these tend to form expansile rather than infiltrative masses[11]. Solid tumors typically lack a significant stromal component and correspond to the medullary type distinguished by Kubo[4]. Others have used yet another term, “cohesive carcinoma”[8], when individual cells or small groups of tumor cells in solid/medullary/cohesive formations lack investment by stroma in contrast to isolated-cell type. Thus, in the Carneiro classification scheme histologic group 1 is isolated-cell, group 2 is gland-forming, group 3 has mixed gland-forming and single cell components, and group 4 is solid. A number of cases remained unclassified by this four-group system due to the scant nature and quality of biopsy material. Tumors with neuroendocrine morphology by H&E were also placed in the unclassified category as well as being considered separately for statistical analysis (see below).

Morphologic features suggesting neuroendocrine differentiation on H&E were evaluated as previously described for neuroendocrine carcinomas, namely, nested/organoid, trabecular, and rosette-forming architecture, relative cellular monotony, eosinophilic cytoplasm, indistinct cellular borders, and evenly dispersed chromatin[3,33,34,47]. Formal mitotic counts were not performed due to a preponderance of small biopsy specimens with limited numbers of high-power fields; however, mitoses were noted in the majority of tumors with neuroendocrine features. Tumors were characterized as signet ring cell carcinomas if >50% of malignant cells were signet ring cells. The degree of chronic inflammatory cell infiltrate within the tumor was graded semi-quantitatively as mild, moderate, or severe. Stromal fibrosis (desmoplasia) was also graded semi-quantitatively as mild, moderate or severe, reflecting a relative proportion of stromal spindle cells to tumor cells.

Histochemistry

EBV encoded RNA *(EBER) in situ* hybridization was performed on paraffin sections (Leica BOND Max system with EBER ASR probe and oligo dT control probe detected using the Bond Polymer AP Red Detection reagents, Leica Microsystems Inc., Buffalo Grove, IL)[48,49] to determine if EBV was localized to malignant epithelial cells. EBV localization to scattered lymphocytes was seen in some EBV-positive and EBV-negative tumor tissues as previously reported[50]. Representative histologic tumor types from all three molecular groups were selected for immunohistochemistry using antibodies to chromogranin A (Leica, 1:200) and synaptophysin (Leica, 1:200). Avidin-biotin complex peroxidase techniques were used

for signal detection on 4 μm sections of formalin-fixed, paraffin-embedded tissues. Antigen retrieval in hot citrate buffer at pH 6.0 was applied before immunostaining.

Statistics

Unsupervised hierarchical clustering revealed the EBV-infected molecular subtype for which EBV localization to malignant cells was proven by *EBER in situ* hybridization. Two additional uninfected molecular classes of gastric cancer were identified on heat maps created using Cluster 3.0 and JavaTreeView software algorithms applied to log2 transformed gene expression data collected on the nCounter system. Genes differentially expressed among the three molecular types of cancer were identified using non parametric Mann-Whitney tests, and p-values were adjusted using Bonferroni correction to account for multiple comparisons. A Bonferroni adjusted p value <0.05 connoted significant differential expression. Box plots show the median and middle two quartiles surrounded by whiskers depicting outliers which are far above or below the interquartile range (IQR) by >Q3+1.5*IQR or <Q1-1.5*IQR, respectively. Associations between discrete histologic parameters and the three molecular types of cancer were evaluated by Fisher's Exact Test or Pearson's Chi-squared test. Statistical analysis was repeated after limiting the cohort to the two uninfected gastric cancer molecular types.

Results

RNA expression profiling reveals three molecular subtypes of gastric adenocarcinoma

The 96-RNA probe test panel that included 73 human mRNAs, 20 viral RNAs, and three controls (Gastrogenus v1™ panel) was applied to 103 macrodissected gastric adenocarcinoma tissues. After data normalization, unsupervised clustering identified three major molecular subtypes, one with high expression of EBV RNAs, and two lacking substantial levels of EBV RNA. The cancers overexpressing EBV RNAs were called the EBV-positive molecular type (n=14), and all 14 were proven to have EBV localized to malignant cells by *EBER in situ* hybridization which is the gold standard assay for defining a tumor as infected [45,50]. Three RNAs involved in the differentiation and signaling of neuroendocrine cells of the gut, *CHGA (chromogranin)*, *GAST (gastrin)*, and *REG4 (regenerating islet-derived family, member 4)*, were significantly overexpressed in one non-infected molecular subtype. Henceforth, the two uninfected molecular subtypes are referred to as Neuroendocrine-high (NE-high, n=34) and Neuroendocrine-low (NE-low, n=55) (Fig 1, Fig S1, Supplemental Digital Content 1, http://links.lww.com/AIMM/A57).

Neuroendocrine RNAs distinguish the three molecular subtypes

Of the 73 human RNAs that were measured, 43 were significantly differentially expressed among the three molecular groups. The 9 human genes more highly expressed in EBV-positive compared with EBV-negative cancers are involved primarily in the anti-viral inflammatory/immune response as previously described[45]. The neuroendocrine markers *CHGA*, *GAST*, and *REG4* were expressed at a lower level in the EBV-positive compared to the NE-high molecular type. Levels of these neuroendocrine markers in the NE-low molecular type were intermediate between those in the EBV-positive and NE-high groups,

suggesting that the EBV-positive tumors had the least amount neuroendocrine differentiation (Fig 1).

Statistical analysis comparing the NE-high and NE-low molecular types revealed differential expression of 32 RNAs (Table 1). Only three genes were upregulated in NE-high compared with the NE-low molecular subtype, and these were the three neuroendocrine markers. The remaining 29 RNAs were downregulated in the NE-high group, and these downregulated RNAs include adhesion factors and intracellular signaling molecules (*ICAM1*, *FSCN1*, *SULF1*, *SPP1*, *INHBA*), and collagen components *COL1A1*, *COL1A2*, and *COL3A1*. Additionally, many inflammatory and immune response genes are downregulated in the NE-high compared to the NE-low molecular types, implicating stromal cells as a factor influencing clustering. The EBV genes *EBER1*, *EBER2*, and *BLLF1* were relatively underexpressed in NE-high compared to the NE-low molecular types, although the levels of these viral RNAs did not approach the elevated levels seen in the EBV-infected carcinomas. Rare EBV-infected stromal lymphocytes could be a source of these viral RNAs in uninfected cancer tissues.

Histologic analysis

H&E stained sections were analyzed to determine histologic classification, to examine stromal content for the extent of chronic inflammation and fibrosis, and to query for morphologic evidence of neuroendocrine differentiation (Table 2). EBV-positive molecular type cancers were noted to have more chronic inflammation compared to NE-high or NE-low tumors and were much more likely to be designated as “gastric carcinoma with lymphoid stroma” (Fig 2). 7/14 EBV-positive cancers received this designation versus 0/34 NE-high and 1/55 NE-low cancers. Tumors with more lymphocytic infiltrate generally showed less stromal desmoplasia, and no EBV-positive molecular type cancers were noted to have severe stromal fibrosis. EBV-positive molecular type cancers were comprised of sheets or large nests of tumor cells with absent or minimal desmoplastic stroma, characteristic of solid carcinoma of the stomach. Focal gland formation was present in some of the otherwise solid-appearing EBV-positive cancers but the focal glands were not associated with stromal infiltration.

There was a statistically significant association between the four histologic Carneiro groups among the three molecular types ($p<0.05$) (Table 2, Fig 3). As described above, EBV-positive carcinomas were predominantly group 4 solid. The NE-high molecular type had a greater proportion of group 1 isolated-cell histology (14/34 cases, 41%) compared to the EBV-positive (0/14) and NE-low type (10/55, 18%), and a greater proportion of signet ring cell carcinomas—11/34 (32%) NE-high tumors were signet ring cell carcinomas compared to 0/14 EBV-positive tumors and 4/55 (7%) NE-low tumors. Classification of tumors into Lauren categories did not have a statistically significant association with molecular subtypes.

Although all gastric cancers in this study were diagnosed as adenocarcinoma rather than neuroendocrine carcinoma, a number of cases were found to have architectural and cytologic neuroendocrine features on H&E stain, such as nested or rosette-forming architecture, cellular monotony, and evenly dispersed chromatin (Fig 4). In addition, some cases had

high-grade neuroendocrine features with increased nuclear/cytoplasmic ratios, hyperchromatic nuclei, and conspicuous mitotic activity. Four biopsy cases additionally showed extensive nuclear molding, single cell apoptosis, and crush artifact, fulfilling the criteria for classic small cell carcinoma (Fig 4E). Two of these small cell carcinoma cases were NE-high and two were NE-low molecular subtype. However, in other cases determination of high-grade vs. low-grade neuroendocrine tumor features could not be made due to the limited size of biopsy material. All tumors with any morphologic neuroendocrine features were separated out for purposes of statistical analysis. While none of the 14 EBV-positive cancers had neuroendocrine features by H&E, such features were found in 4/34 NE-high and 10/55 NE-low molecular type tumors, but this relationship did not reach statistical significance.

Genes associated with histologic subtypes

RNAs differentially expressed among the four Carneiro histologic groups included primarily viral RNAs and those human RNAs involved in the inflammatory/immune response. These RNAs were upregulated in group 4 solid carcinomas which includes the majority of EBV-positive molecular type cancers. Four human RNAs not generally considered part of the inflammatory repertoire that were significantly differentially expressed among the histologic groups are *CHGA* (p=0.007), *PLUNC* (p=0.008), *CYP2W1* (p=0.014), and *PPARG* (p=0.035). *CHGA* was expressed at a higher level in group 1 isolated-cell tumors and group 3 mixed tumors, while *PLUNC* was expressed at higher level in group 4 solid tumors (Fig 5). No RNAs were differentially expressed in association with morphological evidence of neuroendocrine differentiation. Similarly, no RNAs were significantly associated with the Lauren classification or with signet ring cell morphology.

Chromogranin RNA level and protein immunohistochemistry

Given the significant association of the molecular groups with the expression of neuroendocrine RNAs, we assessed neuroendocrine differentiation by immunohistochemistry for chromogranin and synaptophysin in representative tumors from the three molecular types (Table S1, Supplemental Digital Content 2, http://links.lww.com/AIMM/A58). Immunohistochemistry could be performed only on a small fraction of study samples in part because tissue from many biopsy cases had been exhausted for RNA extraction. Immunohistochemistry was attempted in 16 cases with 14 yielding sufficient tissue for analysis, 5 NE-high cases, 7 NE-low cases, and 2 EBV-positive cases. In the NE-high subtype, chromogranin staining was positive in 4/5 cases (1 NE/small cell carcinoma, 2 isolated-cell carcinomas, and one mixed carcinoma). One NE-high case with neuroendocrine morphology was negative for chromogranin but positive for synaptophysin. In the NE-low subtype, chromogranin staining was positive in 1/7 cases (1 isolated-cell carcinoma) while negative in 2 solid carcinomas and 4 NE carcinomas, including two small cell carcinomas. Three of the four chromogranin-negative NE carcinomas were synaptophysin positive. Neither of the two EBV-positive cases had positive chromogranin or synaptophysin staining. No statistically significant correlation between chromogranin RNA level and protein expression could be established (p=0.06).

Neuroendocrine differentiation is observed only in the non-EBV-infected molecular type cancers

Although only a small number of samples were investigated by immunohistochemistry, positive antibody staining for neuroendocrine markers was observed only in non-EBV-infected molecular type cancers. EBV-positive molecular type cancer with solid morphology lacks chromogranin or synaptophysin-expressing malignant cells (Fig 6A-C). In contrast, scattered single tumor cells positive for chromogranin or synaptophysin were seen in NE-high molecular type cancers that were group 1 isolated-cell histology (Fig 6D-F). NE-high tumors with signet ring cells also showed scattered chromogranin or synaptophysin positive cells (Fig 6G-I). NE-low molecular type tumors with isolated-cell histology similarly had neuroendocrine expressing cells. In one such tumor, two differently staining neuroendocrine elements corresponded to two distinct H&E appearances: one with intracellular mucin and signet ring cell appearance and the other with enlarged vesicular nuclei, prominent nucleoli, and eosinophilic cytoplasm (Fig 6J-O). In retrospect, these atypical nuclear features might be consistent with large cell neuroendocrine carcinoma (see Discussion).

Agreement between neuroendocrine differentiation by morphology and immunohistochemistry is imperfect

Several tumors with features of neuroendocrine differentiation assessed by H&E showed at least focal synaptophysin and/or chromogranin staining (Fig 4). These tumors often had multiple morphologies within the same tumor evident even in small biopsy specimens. For example, one synaptophysin-positive case showed areas of tumor forming neuroendocrine rosettes and areas of solid nests (Fig 4A-C). The nuclear features were high-grade in both areas with hyperchromasia and nuclear molding. Several other cases had clearly distinct gland-forming/tubular and nested neuroendocrine components (Fig 4D). A small cell carcinoma that was chromogranin-positive had scattered signet ring cells (Fig 4E-F).

However, the initial morphologic assessment of neuroendocrine features did not always correlate with positive immunohistochemistry for synaptophysin and chromogranin. At least one case considered to be a high-grade neuroendocrine carcinoma by H&E did not show any synaptophysin or chromogranin reactivity (Fig 7A-B). This tumor had nested architecture and two cytologically distinct cell populations, one with finely clumped, salt-and-pepper chromatin and more abundant cytoplasm, and the other with nuclear hyperchromasia, increased nuclear/cytoplasmic ratio, and nuclear molding. Conversely, an NE-low molecular subtype tumor that was initially not noted to have neuroendocrine features on H&E showed synaptophysin positivity. This tumor was classified as a group 1 isolated-cell carcinoma with pleomorphic signet ring cells although with unusually little stromal fibrosis (Fig 7C). On re-examination there were minor areas of trabecular/nested growth composed of eosinophilic non-signet ring cells with hyperchromatic nuclei, indistinct cell borders, and nuclear molding—features of high grade neuroendocrine carcinoma (Fig 7D). Lack of fibrosis and packed appearance of both signet ring cells and eosinophilic cells can also favor classification as solid carcinoma. In our experience, however, solid carcinomas without neuroendocrine features have a different appearance with round cell shapes, well-defined cellular borders, absent nuclear molding, and sheet-like rather than nested growth. Fig 7E shows one such tumor of the NE-low molecular subtype classified as solid (group 4). This

tumor showed no neuroendocrine differentiation by chromogranin or synaptophysin immunohistochemistry.

Discussion

In this study, RNA expression arrays were applied to profile and cluster gastric cancer tissues, which yielded three molecular types of cancer. These three molecular types were distinguished from one another based primarily on EBV status and by the degree of expression of neuroendocrine RNAs. Furthermore, these molecular types correlated in part with histologic features and with histochemical test results for EBV infection and for neuroendocrine differentiation. The EBV-positive molecular type cancers uniformly expressed *EBER* in the malignant cells by *in situ* hybridization. We previously showed that these infected cancers expressed multiple EBV genes as well as a subset of human genes involved in inflammatory response (*TNFSF9, TRAF1, CXCL11, IFITM1* and *FCRL3*)[45]. Overexpression of the B cell markers *MS4A1 (CD20)* and *FCER2 (CD23)*, and the suppressor T cell marker *CD8* are consistent with the chronic inflammatory cell infiltrate in these tumors and their frequent morphologic diagnosis as “gastric carcinoma with lymphoid stroma”. The correlation between the abundant tumor-infiltrating lymphocytes and the molecular evidence of inflammatory response lends validity to the molecular profiling system used in this study.

EBV-positive cancers in this study tended to have solid morphology with minimal fibrosis, features noted by others as well[19,51]. This morphologic type is distinguished by the Carneiro gastric cancer classification but not by the Lauren classification. EBV-positive cancers also had more chronic inflammation in keeping with lymphoid stroma rather than fibrotic stroma. None of the EBV-positive cancers were signet ring cell type, although more cases must be studies to evaluate whether this findings is significant.

A significant finding in the EBV-positive cancers was the lack of neuroendocrine differentiation as evaluated by H&E histology, immunohistochemistry, and RNA expression. Molecular profiling divided the non-infected cancers in this study into two groups that differed from each other in the expression of three RNAs known to be associated with neuroendocrine cell differentiation in the gut, *CGHA, GAST*, and *REG4*[53,54,55]. However, we did not find a significant correlation between RNA expression and neuroendocrine appearance on H&E. There was also no correlation between chromogranin protein positivity assessed by immunohistochemistry and chromogranin RNA expression. Discordance between chromogranin protein and RNA expression has been described in pulmonary small cell carcinomas[56,57,58]. Immunoreactivity for chromogranin is associated with the presence of neurosecretory granules in tumor cells, and many high-grade neuroendocrine carcinomas have absent or aberrant production of such granules. Among primary pulmonary small cell carcinomas, immunopositivity for chromogranin is generally only around 80%[59] and has been reported as low as 23%[60]. Absent or focal chromogranin staining in gastric neuroendocrine carcinomas is also a known phenomenon with reported chromogranin positivity of 40%[61]. The lack of correlation between neuroendocrine RNA expression level and neuroendocrine morphology in this study may be due to 1) the small number of tumors with neuroendocrine morphology, 2) poor reproducibility of

neuroendocrine carcinoma morphologic diagnosis, 3) expression of neuroendocrine RNAs in non-malignant cells, or 4) biopsy sampling may not be representative of the entire tumor. We conclude that while neuroendocrine RNAs seem to be major drivers of molecular subtype in this study, it was not possible to use H&E nor standard immunostain evidence of neuroendocrine differentiation to predict molecular subtype.

In two-way analysis, NE-high and NE-low molecular types differed not only in neuroendocrine related transcripts but also in the expression levels of several other genes involved in intercellular signaling and adhesion as well as inflammation and metabolism. It should be noted that only 73 human RNAs were profiled, and thus there is limited ability to examine pertinent biochemical pathways in these tumors.

Recent progress towards targeted therapy for gastric cancer is refining the way that cancer is classified. Already implemented in routine patient care are tests for *ERBB2(HER2)* protein expression or gene amplification as a means to predict therapeutic efficacy of trastuzumab. In our study, *ERBB2* RNA was expressed in some tumors but it was not differentially expressed among the three molecular types that we delineated[45]. Multiple investigators are pursuing strategies for virus-targeted therapy in the subset of cancers that are EBV-infected[62,63,64,65]. Efforts focused on molecular and histochemical assays will add value to the histopathologic classification of gastric cancer given the inter- and intra-tumoral heterogeneity that confounds histologic evaluation.

Our findings highlight the difficulty in applying neuroendocrine carcinoma designation. Some tumors assessed as having neuroendocrine morphology by H&E were negative for chromogranin or synaptophysin immunohistochemistry. Conversely, some tumors not suspected to have neuroendocrine differentiation showed neuroendocrine marker positivity by immunohistochemistry. Multiple studies have found that neuroendocrine differentiation is under-recognized by morphology alone, and ancillary methods often reveal evidence of it in various tumor types[39,47,55,66]. Large cell neuroendocrine carcinoma is especially difficult to distinguish from poorly differentiated adenocarcinoma because of overlapping cytologic features and growth patterns, and even accumulation of intracellular mucin is permissible in large cell neuroendocrine carcinoma[33,34,35]. In our study we observed inter- and intra-tumoral heterogeneity in tumors with neuroendocrine morphologic features as well.

One important question is: does neuroendocrine differentiation in gastric cancer have clinical implications? It seems clear that tumors fulfilling the diagnostic criteria for small cell carcinoma are a distinct group of biologically aggressive malignancies[67]. Prognostication becomes more difficult when the high grade neuroendocrine component constitutes only part of the tumor. One study set a threshold for tumor designation as large cell neuroendocrine carcinoma if the neuroendocrine component was >50% of the tumor by chromogranin immunostain[33]. In that study, adenocarcinomas with 20 to 50% chromogranin-positive large cell neuroendocrine component showed reduced survival compared to adenocarcinoma with no neuroendocrine component. There have been conflicting reports of a worse[37,39] or better[41,68] prognosis associated with any focal neuroendocrine differentiation by immunohistochemistry. Given the uncertainty of the morphologic criteria and the variability of protein immunohistochemistry, it seems logical to

ask if an RNA-based molecular test may provide a more objective measure of neuroendocrine differentiation. Further studies are necessary to test if an RNA threshold is clinically significant, and to compare the performance of RNA-based tests to protein immunohistochemistry. It is likely that tumors with a clinically significant level of neuroendocrine differentiation would be defined not by a single RNA but by a panel of several RNA markers such as the ones used in this study. An important limitation of this study is the lack of outcome data to determine if the three molecular subtypes correspond to gastric cancer subsets with clinically significant differences in disease progression or response to therapy.

In conclusion, our study shows molecular evidence of neuroendocrine differentiation in over a third of gastric adenocarcinomas. However, EBV-infected tumors completely lack neuroendocrine differentiation either in their molecular profile or by morphology. EBV-infected are predominantly solid morphologic type which is distinguished by the Carneiro histologic classification but not the Lauren classification. Our study also confirms that molecular profiling has promise as a robust tool, applicable in small fixed biopsies, to add value in classifying gastric cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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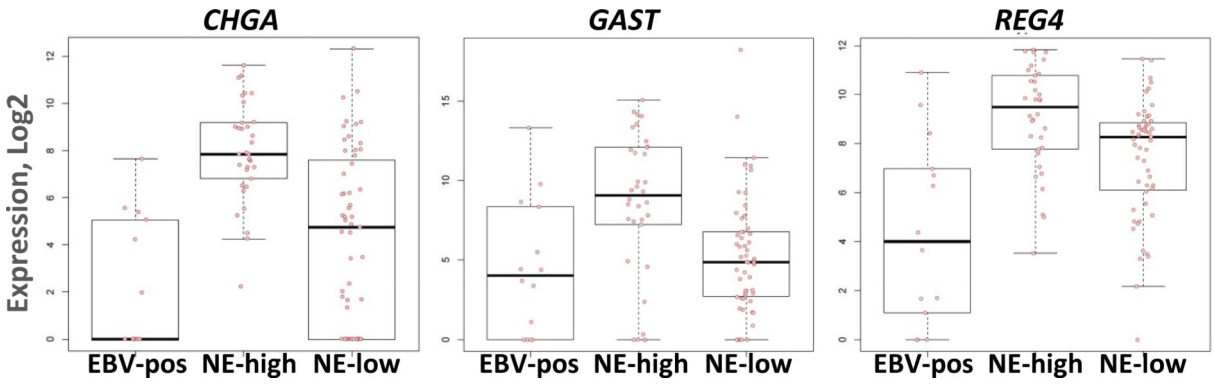

Figure 1. Differential expression of neuroendocrine (NE) RNAs by molecular subtype of gastric cancer

CHGA (chromogranin), *GAST (gastrin)*, and *REG4* are expressed more highly in NE-high molecular type cancers compared to EBV-positive or NE-low molecular types.

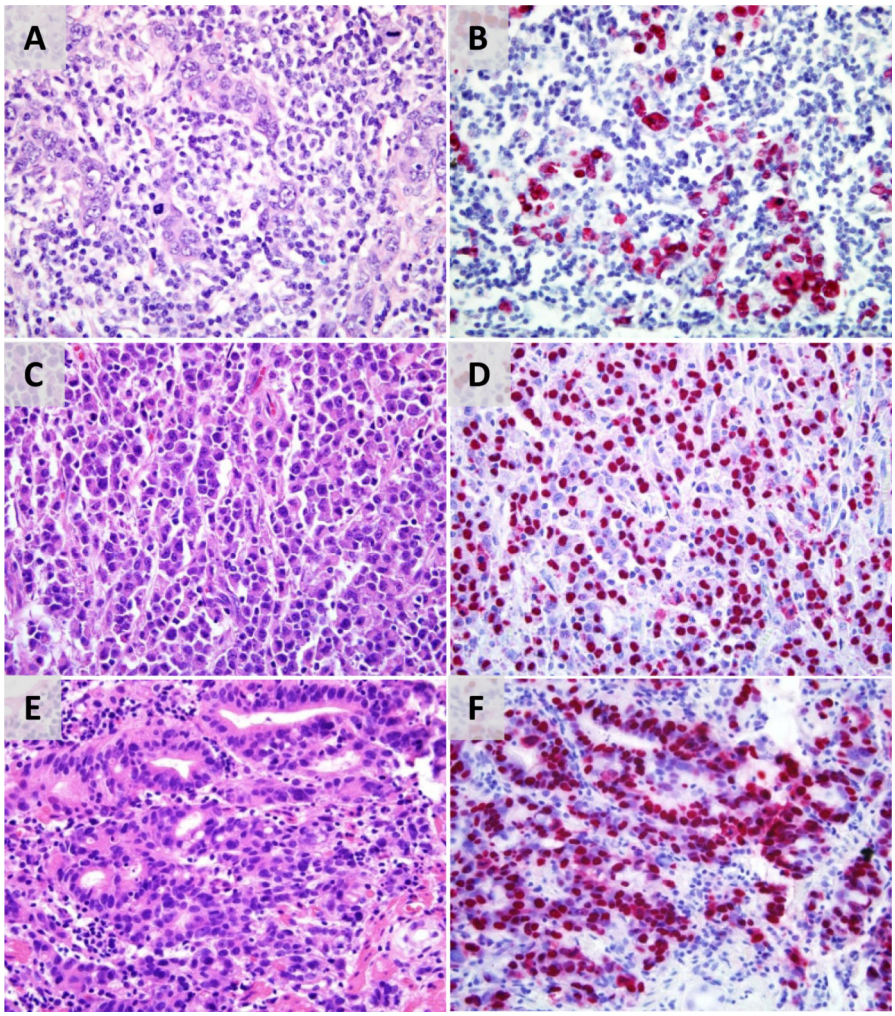

Figure 2. Morphologic range of EBV-positive gastric adenocarcinomas

(A) Gastric cancer with lymphoid stroma has undifferentiated epithelial nests surrounded by a lymphoplasmacytic infiltrate with lack of stromal desmoplasia; (B) *EBER* RNA is expressed in the malignant epithelial cells. (C) Solid carcinoma is a sheet-like proliferation of malignant epithelial cells with deep eosinophilic cytoplasm and virtually no intervening fibrous or lymphoid stroma; (D) *EBER* RNA is expressed in the malignant epithelial cells. (E) Solid carcinoma with areas of gland formation in a lymphoid stroma without significant fibrosis; (F) *EBER* RNA is expressed in the malignant epithelial cells.

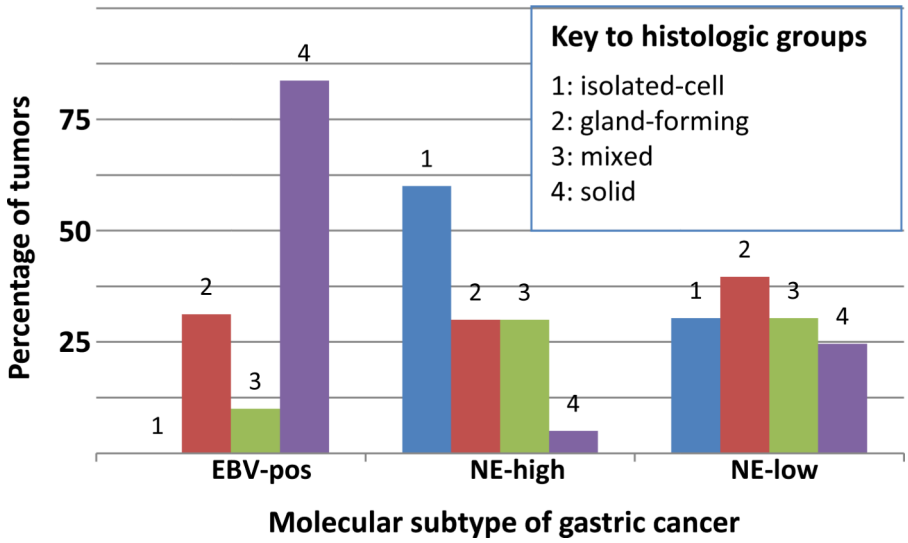

Figure 3. Distribution of the four histologic groups (relative proportions) among the three molecular types

EBV-positive carcinomas are predominantly group 4 solid while very few of the NE-high molecular type tumors are classified in that group. Instead, the NE-high molecular type is enriched for tumors having group 1 isolated-cell histology while none of the EBV-positive cancers are classified as isolated-cell morphology ($p<0.05$, Chi-square test).

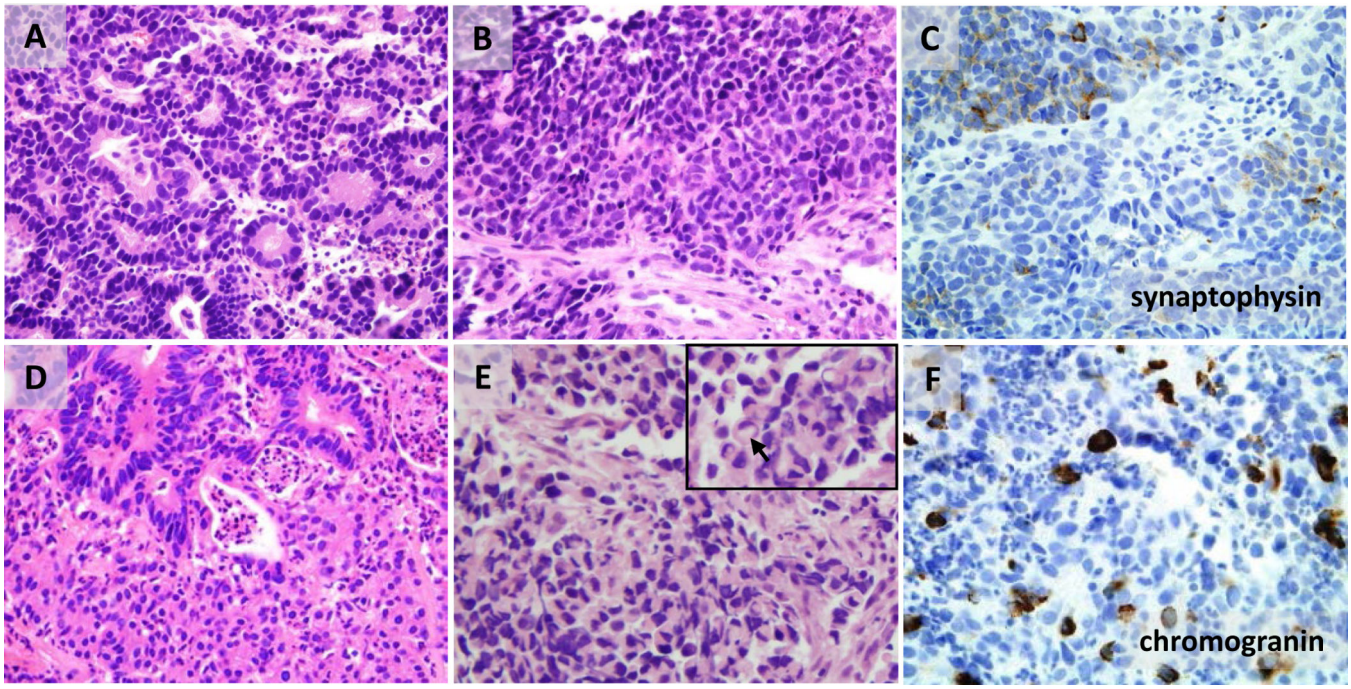

Figure 4. Morphologic heterogeneity of carcinomas with neuroendocrine features

A carcinoma with high-grade neuroendocrine nuclear features such as hyperchromasia and nuclear molding has areas of rosettes (A) and solid nests (B) and shows focal synaptophysin positivity in both areas (C, only nested area is shown). (D) A neuroendocrine tumor with two intermingled morphologies, visible as gland-forming and solid areas, demonstrates cellular monotony, unapparent cellular borders, and evenly dispersed chromatin in both morphologic components but more ample eosinophilic cytoplasm in the solid nests. (E-F) Small cell carcinoma with nuclear molding and crush artifact has rare cells with signet ring morphology (E, arrow in inset) and is focally positive for chromogranin (F).

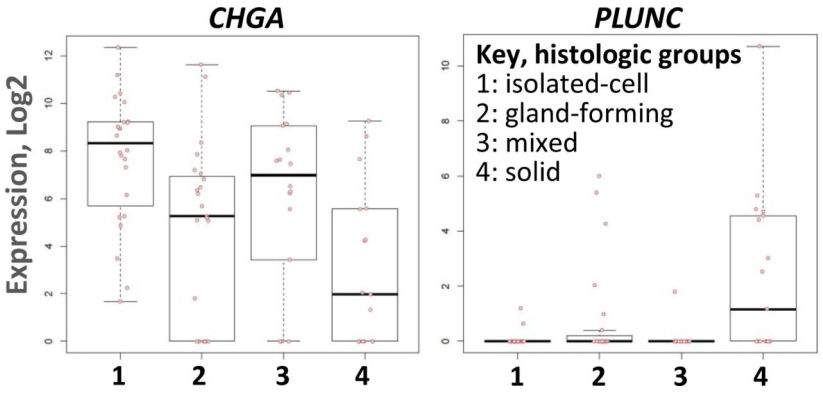

Figure 5. Differential expression of *CHGA* and *PLUNC* by Carneiro histologic groupings

CHGA is expressed at higher levels in histologic groups 1 and 3 while *PLUNC* is expressed at a higher level in histologic group 4.

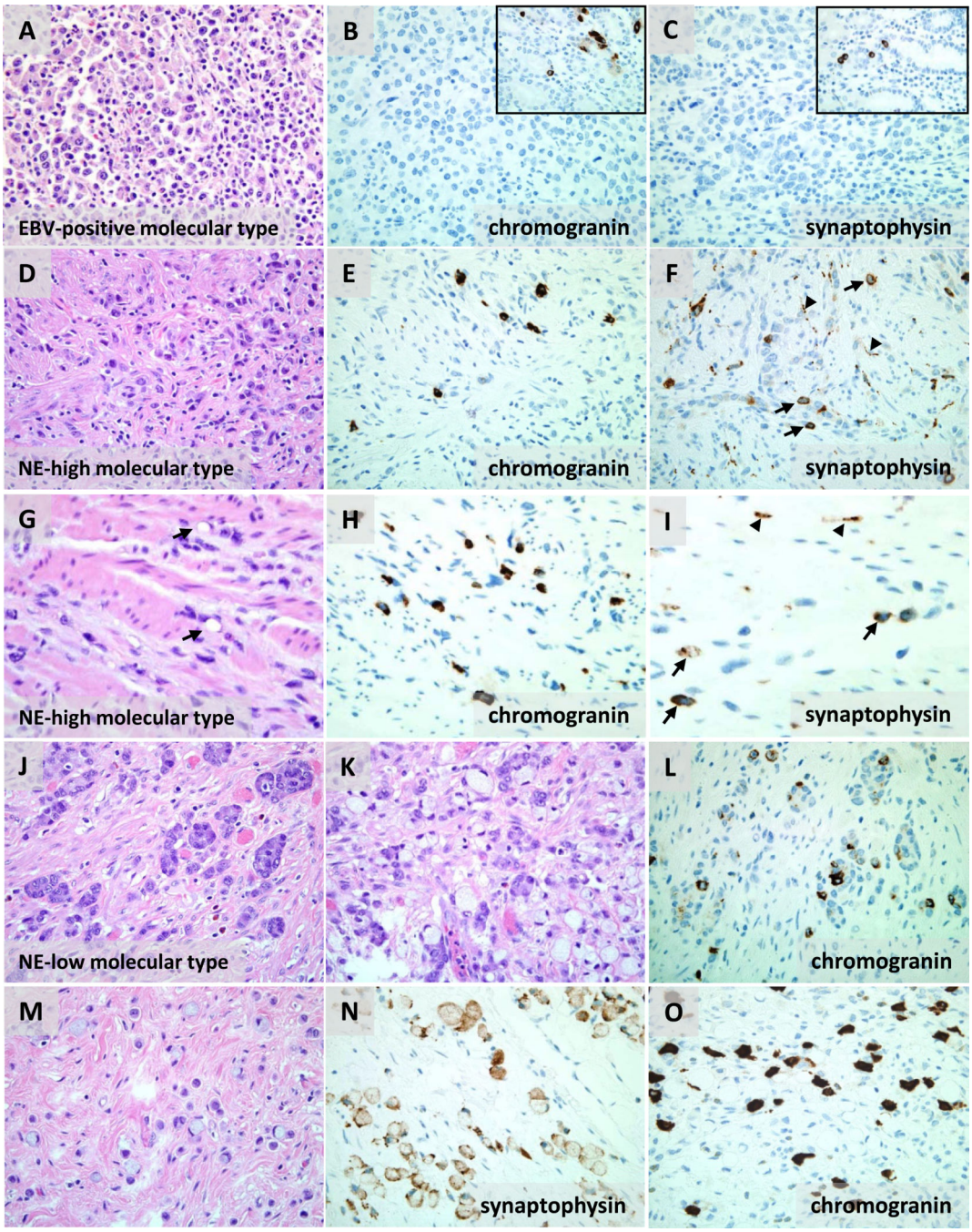

Figure 6. Neuroendocrine differentiation is observed in diffuse cancers of the NE-high and NE-low molecular types but not in the EBV-positive type

(A) Solid carcinoma of EBV-positive molecular type is negative for chromogranin (B) and synaptophysin (C) with positive internal control in the neuroendocrine cells of residual non-neoplastic gastric pits (insets in B and C). (D) A carcinoma of NE-high molecular type and infiltrating isolated-cell histology has scattered individual malignant cells expressing chromogranin (E) and synaptophysin (arrows, F). (G) Similarly, a signet ring cell carcinoma of NE-high molecular type has chromogranin (H) and synaptophysin expressing cells (arrows, I). Synaptophysin also highlights small nerve twigs within the abundant fibrous

stroma (arrowheads, F and I). (J-O) An infiltrative carcinoma of NE-low molecular type has focal areas of small nests and short cords of cells with enlarged vesicular nuclei, prominent nucleoli, and eosinophilic cytoplasm (J), other focal areas in which the nests of eosinophilic cells have admixed signet ring cells (K), but predominantly the tumor consists of eosinophilic and signet ring cells infiltrating singly (M). All three of these tumor morphologies are embedded in dense fibrous stroma. Nested areas have individual chromogranin positive cells within the nests (L). The singly infiltrating eosinophilic cells are chromogranin positive and synaptophysin negative while the signet ring cells have the reverse pattern—chromogranin negative and synaptophysin positive (N,O).

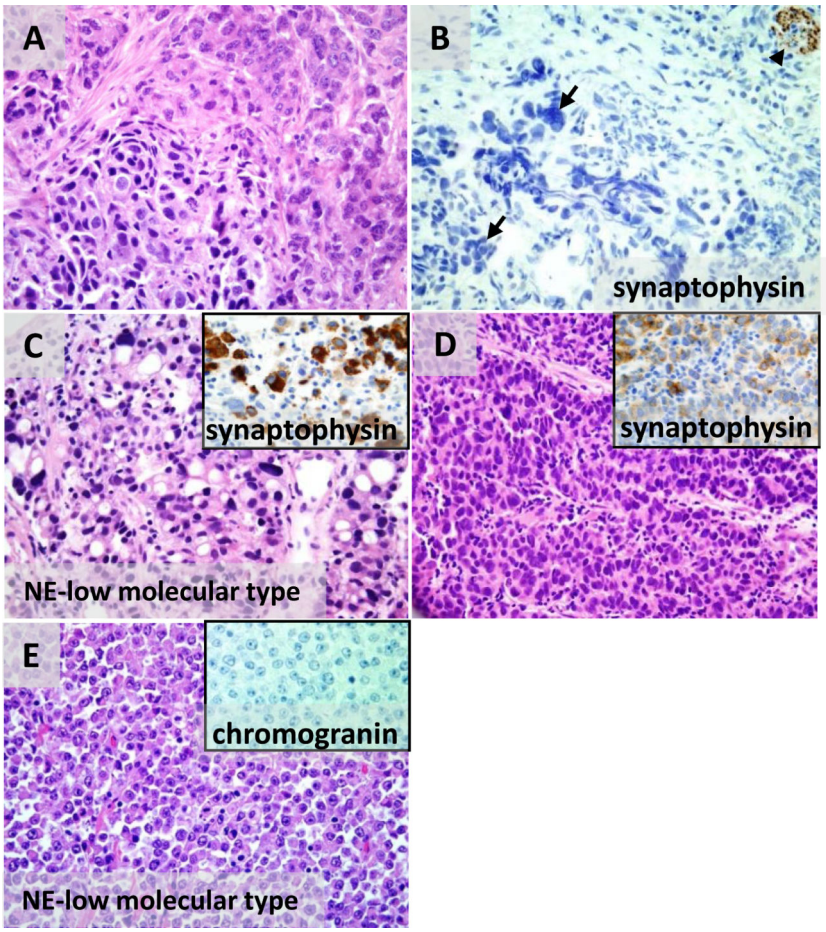

Figure 7. Agreement between neuroendocrine differentiation by morphology and immunohistochemistry is imperfect

(A-B) A tumor with two distinct neuroendocrine cytologic patterns on H&E, salt-and-pepper chromatin (A, upper right) and hyperchromasia and nuclear molding (A, lower left) is negative for synaptophysin (B, arrows at tumor cells with crush artifact) with positive internal control synaptophysin staining in the stromal nerve (B, arrowhead). The tumor cells are also negative for chromogranin protein (not shown). (C-D) An NE-high molecular subtype carcinoma has spatially separate zones of pleomorphic signet ring cells (C) and trabecular/solid nests of non-signet ring cells with high grade neuroendocrine morphology (D). In both zones the malignant cells express synaptophysin (insets in G and H). (E) A carcinoma of NE- low molecular type and solid histology has well-defined cellular borders, prominent nucleoli, absent nuclear molding, and sheet-like growth, and is negative for chromogranin (inset) and synaptophysin (not shown).

Table 1

RNAs significantly differentially expressed in NE-high and NE-low molecular types of gastric adenocarcinoma[a]

Gene symbol	Colloquial name	Description or function	Direction of difference[b]
SULF1		Cell signaling, sulfatase	↓
CHGA	chromogranin	Neuroendocrine cell, gastrin signaling	↑
SPP1		Osteogenesis, secreted phosphoprotein	↓
FSCN1		Cell morphology and motility	↓
TNFSF9		Antigen processing, TNF ligand cytokine	↓
INBA	inhibin	Hormonal regulation and cell growth	↓
FCGR2B		Phagocytosis and antibody production	↓
PTGS2	COX2	Prostaglandin and gastrin signaling	↓
GAST	gastrin	Neuroendocrine cell, stimulation of acid secretion	↑
ICAM1		Cell adhesion	↓
SLC2A1	Glut 1	Glucose transporter	↓
CD4		Helper T cells, MHC class II antigen processing	↓
CD70		TNF ligand, T and NK cell activation	↓
DKK4		Embryonic development	↓
SERPINH1		Collagen synthesis, peptidase inhibitor, heat shock	↓
TRAF1		TNF receptor	↓
CXCL1		Immune development and homeostasis	↓
IGLL1	CD179b	B cell growth	↓
SPARC	osteonectin	Protects from apoptosis, docetaxel response	↓
REG4		Neuroendocrine cell, regenerative islet-derived family	↑
COL1A1		Type I collagen component	↓
EBER1		Non-coding viral RNA	
GPR183	EBI2	G-protein coupled receptor, EBV-induced	↓
EBER2		Non-coding viral RNA	
COL1A2		Type I collagen component	↓
BLLF1		Viral RNA, entry via CD21 receptor	
HIF1A		Systemic response to hypoxia	↓
TYMS		Thymidilate synthase, 5FU response	↓
THY1		Control of inflammatory cell recruitment	↓
BCL2L11	BIM	Activator of apoptosis, BCL2-like	↓
COL3A1		Type III collagen component	↓

[a] FDR adjusted P values are <0.05 for all RNAs listed in the table; RNAs are ranked with most significant P values near the top

[b] Up arrows indicate RNAs expressed at a higher level in NE-high compared to NE-low subtype, and down arrows indicated RNAs expressed at a lower level in NE-high compared to NE-low subtype.

Table 2

Histopathologic features in three molecular subtypes of gastric adenocarcinoma

		Molecular Subtype				
Histologic feature	**# of cases, n=103**	**EBV-positive n=14**	**NE-high n=34**	**NE-low n=55**	**3-way P value[d]**	**2-way P value[e]**
Chronic inflammation					<0.0001[f]	<0.0001
Mild	42	0	18	24		
Moderate	45	5	14	26		
Severe	16	9	2	5		
Stromal fibrosis					0.0008	0.0001
Mild	29	11	5	13		
Moderate	47	2	15	30		
Severe	12	0	6	6		
Indeterminate[a]	15	1	8	6		
Lauren classification					0.3[f]	0.67
Intestinal	30	3	11	16		
Diffuse	36	6	15	15		
Unclassified[b]	37	5	8	24		
Carneiro histologic groups[c]					0.0003[f]	0.0002[f]
1: isolated-cell	24	0	14	10		
2: gland-forming	23	3	7	13		
3: mixed	18	1	7	10		
4: solid	17	8	1	8		
Unclassified[a,b]	21	2	5	14		
Neuroendocrine morphology					0.25	0.2
Yes	14	0	4	10		
No	89	14	30	45		
Gastric cancer with lymphoid stroma					<0.0001	<0.0001
Yes	8	7	0	1		
No	95	7	34	54		
Signet ring cell carcinoma					0.002	0.12
Yes	15	0	11	4		
No	88	14	23	51		

[a] Due to limited nature of biopsy material

[b] Unclassified cases include tumors with neuroendocrine morphology

[c] Based on Carneiro histopathologic classification

[d] Statistical analysis of all three molecular subtypes

[e] Statistical analysis of the EBV-positive and EBV-negative (combined NE-high and NE-low) cancers

[f] Determined by Chi-square test; all other P values determined by two-tailed Fisher's exact test