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ORIGINAL ARTICLE

The contribution of cell phenotype to the behavior of gastric cancer

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Abstract
Background Several histochemical studies suggest a role of tumor cell phenotype and related differentiation markers in the prognostic assessment of gastric cancer. Unfortunately, most studies have dealt with single or a few markers and have paid limited attention to their interplay with tumor histological types, which are potentially informative of prognosis.
Methods In this study, 292 invasive (T1b to T4) gastric cancers with prolonged follow-up and carefully analyzed histotype, inclusive of histotype-based grade, were investigated histochemically with a panel of 14 phenotypic markers known to be expressed in normal gut tissues and gastric cancer.
Results Three of seven intestinal type markers investigated showed a trend for improved prognosis, one of which, CDX2, was stage independent. Three among gastric and pancreatobiliary duct markers (MUC1, MUC6, and pepsinogen II), predicted more severe prognosis stage independently, as did a combination of eight potentially informative ($p < 0.1$ at univariable Cox analysis) markers. Cancers with predominantly intestinal phenotype had significantly better prognosis than those with predominantly gastric, mixed, or poorly defined phenotypes; among the latter, those with high lymphocyte response, with favorable outcome, were separated from anaplastic cancers, with ominous prognosis. At multivariable analysis, CDX2 and the eight marker combination proved to be stage- and grade-independent predictors.
Conclusions When individually considered, and with the exception of CDX2, the biomarkers investigated gave an appreciable, although moderate, contribution to the prognostic evaluation of gastric cancer. Combined analysis of all potentially informative markers gave more important information, highly additive to both stage and histotype-based grade.

Keywords Tumor cell phenotype · Phenotypic markers · Histotype · Prognosis · Gastric cancer

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Introduction

Tumor cell phenotype, which has proven to be predictive of natural history and prognosis in various neoplasms, can be expected to contribute substantially to the diagnosis of gastric cancer, which is known to encompass a variety of histological patterns often difficult to evaluate. Histochemical and ultrastructural studies have documented the plurality of tumor cell phenotypes occurring in gastric cancer, ranging from gastric foveolar-superficial and mucopeptic (pyloric gland or mucous-neck) cells to intestinal goblet cells or absorptive columnar enterocytes, in addition to infrequently reported gastric parietal or chief cells,

intestinal Paneth cells and various kinds of metaplastic hepatopancreatic or endocrine components [1–3].

Among the phenotype-associated markers more extensively investigated we find secretory products such as mucins (including gastric foveolar cell mucin HGM or MUC5AC, mucopeptic cell MUC6, goblet cell MUC2 and CAR5, gastric and pancreatobiliary duct marker MUC1 [2, 4–8]), or enzymes (such as pepsinogen II for mucopeptic cells, lysozyme for intestinal Paneth cells, cathepsin E for foveolar cells, and neutral carboxypeptidase CD10 for absorptive enterocytes [4, 9, 10]). The transcription factor CDX2, cadherin 17 (CDH17) and cytokeratin 20, as intestinal phenotype markers, and cytokeratin 7, as a pancreatobiliary duct marker, have also been widely tested [11–13].

Investigation of phenotypic markers, mostly restricted to individual or to a limited panel of markers, has given interesting histogenetic, diagnostic, and, to some extent, prognostic information, although sometimes with contradictory findings. This evaluation has, however, also highlighted the modest phenotypic background of commonly used histological classifications. In particular, in Laurén's classification [14] the so-called intestinal histotype often failed to show a truly intestinal phenotype [1, 2, 6] or gene expression profile [15, 16]. Of interest, separation of intestinal from gastric, mixed, or undetermined (unclassified or null) phenotype may provide useful, stage-independent prognostic information [3, 7, 9, 11, 13, 17, 18].

In parallel investigations, several histological types and subtypes of prognostic value have been identified [19–23] that showed correlation with number and severity of genomic alterations [24] and permitted the development of a simple grading system, predictive of patient survival [25]. The system proved highly effective for a substantial proportion (about 30 %) of tumors fitting in the low- or high-grade groups, although leaving the intermediate-grade group largely unpredictable [26]. The precise relationship of such histotype-based grading with cell phenotype remains to be ascertained.

In the present study we simultaneously applied a wide panel of phenotype-linked cellular markers, inclusive of most among those found to be predictive in separate individual studies, to a large series of gastric cancers, to search for any relationship between individual markers, combinations of markers, or tumor cell phenotype with histotypes, and clinicopathological parameters predictive of tumor behavior and actual patient survival.

Materials and methods

Case series

Among invasive (T1b to T4) gastric cancers resected at the San Matteo Hospital in Pavia or at the San Martino University Hospital in Genoa between 1984 and 2000, 292 cases with extensive tumor sampling and carefully assessed TNM stage were selected [26]. Their histopathological and clinicopathological patterns have already been described [25]; 2 of the original 294 cases had to be excluded because there was no remaining tumor tissue in the paraffin block. No antiblastic therapy had been administered. Nineteen cases died perioperatively (within 1 month from surgery); by the end of December 2009, patients had either died of the disease (166) or other causes (51) or were alive (56) after being followed for at least 9 years. A median follow-up of 13 years (25th–75th = 7–18) was calculated.

New serial sections were taken from representative paraffin blocks of each tumor and stained with hematoxylin and eosin and Alcian blue–periodic acid-Schiff (PAS) or with immunoperoxidase [25] using specific antibodies directed against 14 different markers that are potentially informative of tumor cell phenotype, including MUC2 (Ccp58; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MUC5AC (CLH_2; Novocastra, UK), Bara's M1 antibodies [4, 5], MUC6 (CLH5; Novocastra), MUC1 (VU4H5; Santa Cruz Biotechnology), cytokeratin 7 (OV-TL12/30; DAKO, Denmark), cytokeratin 20 (K20.8; DAKO), CDX2 (EPR2764Y; Cell Marque, Rocklin, CA, USA), CDH17 (3H2; Abnova, Taiwan), CD10 (56C6; Novocastra), cathepsin E (C-20; Santa Cruz), lysozyme (A 099; DAKO), LGALS4 (1E8; Sigma Aldrich, St. Louis, MO, USA), pepsinogen II [9], and CAR5 [6]. Because of progressive exhaustion of tumor tissue in some blocks and other technical problems, the number of cases in which the expression of individual markers was successfully assessed ranged from a minimum of 246 to a maximum of 289.

The evaluation of each marker was essentially based on the proportion (%) of tumor cells showing positive staining, irrespective of its intensity and intracellular distribution, be it cytoplasmic, membranous, or nuclear (except for the exclusively nuclear localization of the transcription factor CDX2). Separate evaluation of membrane staining only was added in the case of CDH17, whereas for LGALS4 (Galectin 4) separate nuclear staining evaluation was added and compared with the M30 antibody test (M30 clone; Roche Diagnostics, Basel, Switzerland) for apoptosis. For both CDH17 and LGALS4, an evaluation based on intensity staining score (0–3) was also tested. Each slide was evaluated independently by at least two pathologists, followed by joint revision of discrepant cases until consensus was reached. For most markers, a 10–20 % expression cutoff was chosen, depending on their median expression value, so as to obtain balanced positive and negative groups, as far as possible.

To investigate the relationship between tumor phenotype and histotype, gastric cancers were first classified into five main types, including cohesive (differentiated or

glandular and solid), diffuse and mucinous cancers according to commonly used histological criteria [14, 27, 28], to which lymphocyte-rich tumors with predominance of CD8 (C8/144b clone, DAKO) reactive cells [26], and the anaplastic cancers [23, 25, 26, 28, 29] were added. Alternatively, nine prognostically informative subtypes of more recent characterization were considered, such as muconodular (mucinous cancer with expansile growth) [22], well-differentiated tubular [21, 25] and diffuse cancer with entrapping desmoplasia [23], which, together with the high CD8-positive lymphocyte response (HLR) cancer [19, 20, 26], formed the low grade (grade 1) group. Ordinary cohesive, diffuse, and mucinous cancers formed the intermediate grade (grade 2) group, and highly invasive mucinous and anaplastic cancers [23, 26] formed the high grade (grade 3) group in a system shown to provide stage-independent prognostic information [25, 26].

Statistical analysis

Data distribution was expressed as counts and percent for categorical variables and was compared by means of the Fisher exact test. The Spearman *rho* correlation coefficient was used to measure the association of variables on a continuous scale. Follow-up extended from the date of surgery to the date of death from gastric cancer or to the last available assessment. Patients dying of other causes were censored at the date of their death. Median follow-up and its interquartile range (25th–75th) was computed by means of the inverse Kaplan–Meier method. Cox regression was used to assess the prognostic role of a series of biomarkers. Univariable, bivariable (with stage), and multivariable models were fitted. Clinically relevant variables were included in the multivariable model in addition to stage, provided they were not collinear. Hazard ratios (HR) and their 95 % confidence intervals (95 % CI) were computed. The proportional hazard assumption was tested, based on Schoenfeld residuals. Death rates per 100 person-years (95 % CI) were also computed as summary measures. For assessing model performance, we also calculated the Royston explained variation and Harrell's *C* statistic for discrimination. Finally, for each patient, a "biomarker burden" was computed as a linear combination of all the biomarkers (dichotomized as in supplementary Table 2) with $p < 0.1$ at univariable survival analysis; each marker was weighted by the beta coefficient derived from a univariable Cox model to account for its contribution to the outcome. The tertiles of the distribution of computed biomarker burdens were included in a bivariable analysis together with stage and in a multivariable analysis also inclusive of histotype-based grade. The linearity of risk across tertiles of biomarker burden was tested with a likelihood ratio test. All tests were two sided. Stata 12 (StataCorp, College Station, TX, USA) was used for computation.

Results

Prognostic value of individual or combined markers

The phenotypic profile of the 14 markers used was checked first on normal gastrointestinal mucosa and pancreatobiliary ducts as outlined in supplementary Table 1. Notably, cathepsin E and lysozyme tended to expand their reactivity to cells normally unreactive (e.g., goblet and mucopeptic cells), when involved in inflammatory processes (inducible markers).

Based on the foregoing findings, the markers were phenotypically categorized as intestinal, gastric, pancreatobiliary, or inducible. As reported in Table 1, at univariable survival analysis all intestinal-type markers, with the exception of CD10, showed a trend for favorable prognostic influence; however, only CDX2 survived stage-inclusive bivariable analysis. On the other hand, the gastric mucopeptic cell markers PGII and MUC6 as well as the broader gastric and pancreatobiliary marker MUC1 showed a negative prognostic influence, which was retained, even if weakened, at stage-inclusive bivariable analysis (Table 1). None of the remaining markers showed significant prognostic influence. In the case of CDH17 and LGALS4, no significant survival difference was observed when the intensity score of reactivity rather than the proportion of reactive cells, or the membranous rather than cytoplasmic, nuclear, or total reactivity were considered. Nuclear LGALS4 was prominent in some goblet cells floating in mucin lakes; up to 30 % of such cells proved reactive for the apoptosis marker M30 (Fig. 1).

In an alternative analysis based on the combined effect of the eight markers with $p < 0.1$ at univariable analysis (see Table 1), the "biomarker burden" was calculated as reported in supplementary Table 2. It appeared to be an independent predictor of risk in a bivariable analysis inclusive of stage (stage-adjusted HR: 1.53, 95 % CI 1.24–1.89, $p < 0.001$). Moreover, a linear increase in risk over tertiles was shown (stage-adjusted HR: 1.64, 95 %CI 1.30–2.07, $p < 0.001$) with both a high Harrell's *C* statistic of 0.78 and Royston explained variation of 0.54; pertinent Kaplan–Meier curves are shown in Fig. 2.

Cancer phenotypes and their prognostic value

An association was found in tumors between markers normally expressed by the same cell type (e.g., between MUC6 and PGII, both from mucopeptic cells: Spearman's *rho* 0.36, $p < 0.001$) and between markers of different cells

Table 1 Survival analysis of gastric cancer patients as a function of tumor marker expression

Marker	Association with CDX2, rho[a]	Expression cutoff (%)	Cases positive/total (%)	HR	95 % CI	*p* value	
						Univariable	With stage
Intestinal							
CDX2	1.00	>20	152/257 (59)	0.53	0.39–0.73	<0.001	0.001
Cytokeratin 20	0.39	≥10	71/262 (27)	0.72	0.49–1.05	0.091	0.173
MUC2	0.43	>10	120/282 (43)	0.71	0.51–0.98	0.037	0.446
CAR5	0.32	>10	146/289 (51)	0.71	0.54–1.01	0.054	0.093
CDH17	0.46	>10	128/250 (51)	0.80	0.58–1.11	0.177	0.574
LGALS4	0.30	>30	123/246 (50)	0.67	0.49–0.93	0.017	0.284
CD10	0.26	≥10	60/254 (24)	0.87	0.60–1.28	0.486	0.688
Gastric							
MUC5AC	−0.11	>10	133/288 (46)	0.94	0.69–1.27	0.676	0.742
MUC6	−0.11	≥10	62/249 (25)	1.49	1.04–2.14	0.029	0.043
PGII	−0.12	≥10	82/289 (28)	1.52	1.10–2.09	0.011	0.078*
MUC1	−0.36	>20	121/282 (61)	1.61	1.16–2.24	0.004	0.033
Pancreatobiliary							
Cytokeratin 7	−0.13	>20	115/258 (45)	1.29	0.94–1.79	0.117	0.229
Inducible							
Cathepsin E	−0.29	>20	164/278 (59)	0.97	0.70–1.33	0.833	0.148
Lysozyme	−0.28	>10	125/264 (48)	0.88	0.64–1.21	0.420	0.916

* $p = 0.037$ with ≥5 % cutoff

[a] Spearman's *rho* of marker association with CDX2, taken as reference

from the same tissue (e.g., MUC5 and MUC6, from gastric foveolar or mucopeptic cells, respectively: *rho* 0.34, $p < 0.001$). In general, intestinal markers showed a trend for positive association with each other and for an inverse association with gastric, pancreatobiliary or inducible markers (Table 1). It should be noted that, in the investigated cancers, lysozyme, despite its normal expression by intestinal Paneth cells, was positively associated with gastric and negatively associated with intestinal markers, a behavior likely reflecting its usual expression in normal and inflammation-associated gastric mucous-neck cells.

In Table 2, tumors have been grouped according to the tissue phenotype suggested by the differentiation markers they expressed, so as to obtain a predominantly intestinal (50 % or more tumor cells reactive for MUC2, CAR5, CDH17, and/or CD10, with <25 % cells reactive for MUC5, MUC6, MUC1, and/or PGII), predominantly gastric (>50 % cells reactive for MUC5, MUC6, MUC1, and/or PGII with <25 % reacting for MUC2, CAR5, CDH17, and/or CD10), mixed (≥25 % reactivity for both gastric and intestinal markers), or poor (<25 % tumor cells reactive for both gastric and intestinal markers) phenotype. Prognostic analysis of the four tumor groups showed a more favorable behavior of the intestinal compared to the remaining phenotypes. Of interest, two distinct histotypes, the anaplastic and HLR, with very different prognosis, accounted for most (31/34) of the poorly reactive phenotype group, with its anaplastic subset showing significantly worse prognosis than all remaining tumors and its HLR subset significantly better prognosis than all other tumors apart from those showing an intestinal phenotype.

Based on the results outlined in Table 1, CDX2 was chosen as a prototype intestinal marker with stage-independent favorable prognostic influence and MUC1 as a gastric (and pancreatobiliary) marker with unfavorable influence. Their combined analysis in 255 cases (Table 3) showed a clearly improved prognosis for tumors with an excess of CDX2 over MUC1 (group a), compared either to those with a predominance of MUC1 (group d) or to those with poor expression of both markers (group c).

Distribution of markers among histological types, subtypes, and grades

In general, no specific association of a tumor phenotype with a distinctive histological structure was observed, although a trend for predominance of cohesive (mostly glandular) forms among intestinal phenotype and of diffuse or mixed forms among gastric phenotype cancers was noted. Most markers showed unequal distribution among the five main histotypes considered (Table 4A).

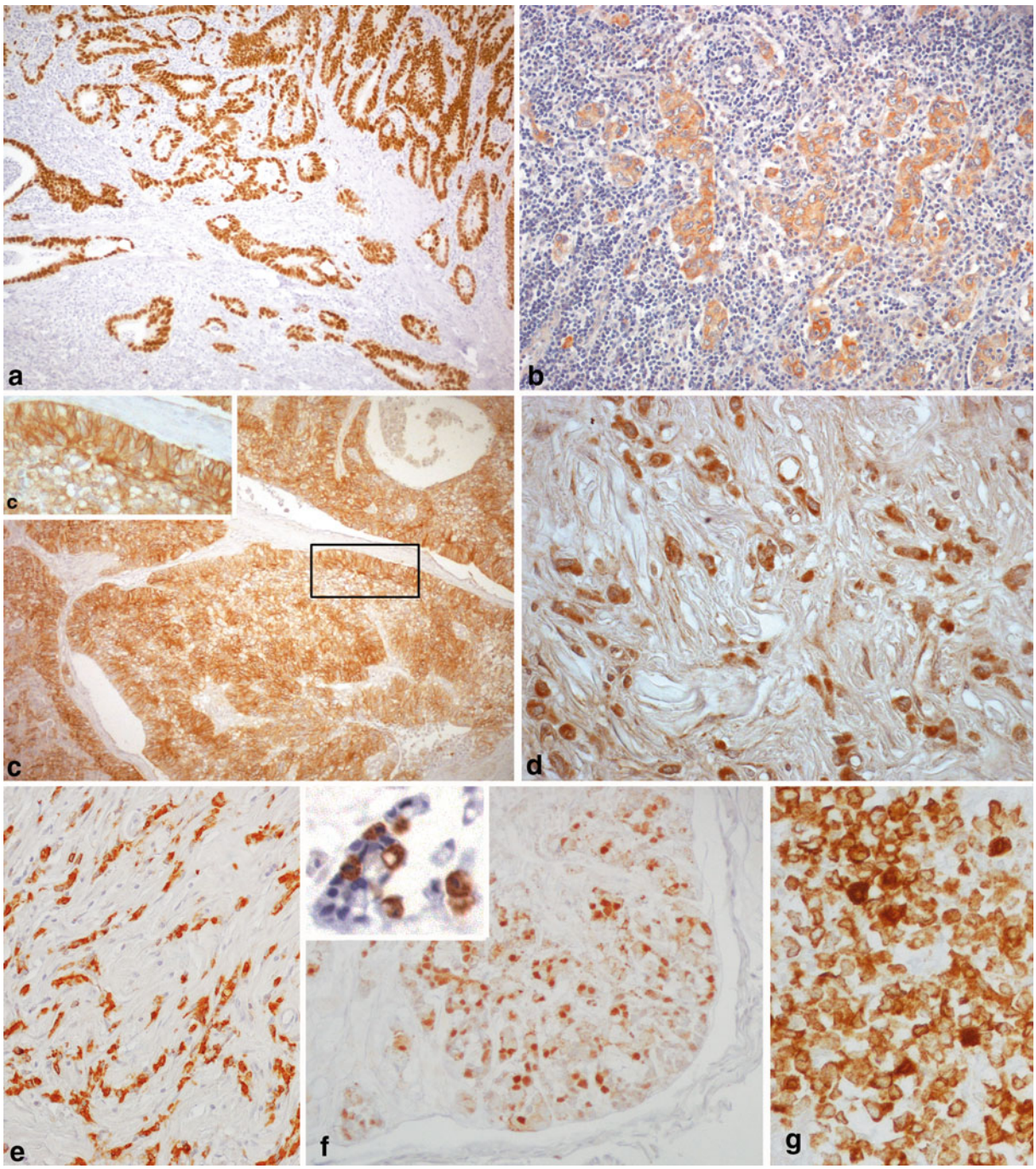

Fig. 1 Immunoperoxidase stained sections of gastric cancers: **a** CDX2-reactive well-differentiated tubular cancer (grade 1) invading the submucosa from its intramucosal (*upper right*) site of origin. **b** Intense cathepsin E reactive epithelial tumor nests in an EBV positive lymphocyte-rich HLR cancer (grade 1). **c** CDH17 positive papillary cancer (ordinary cohesive, grade 2) of intestinal phenotype (also reactive for CDX2, LGALS4, and MUC2), somewhat mimicking small intestine villi. In the *inset* **c**, the columnar epithelium bordering the lumen is enlarged to show membranous staining. **d** PGII reactive ordinary diffuse cancer (grade 2) of gastric phenotype (also stained with MUC6). **e** CAR5 reactive, intestinal phenotype spindle cells of a diffuse desmoplastic, grade 1 cancer embedded in fibroblast-rich stroma. **f** LGALS4 positivity (mostly nuclear) in a grade 1 muconodular cancer of intestinal goblet cell phenotype, also reactive for CDX2, MUC2 and CD10. Note, in *inset*, M30 immunoreactivity of some cells in a consecutive section. **g** MUC1-positive, large cell, non-neuroendocrine anaplastic cancer (grade 3). **a**, ×100; **c** ×100 (*inset* ×400); **b** ×200; **d** ×400; **e** ×100; **f** ×140 (*inset* ×400); **g** ×400

Differences of interest were (1) the high reactivity, as expected, of mucinous cancers for MUC2 and MUC5AC, although not for MUC6 and MUC1 or for enzymes such as lysozyme, cathepsin E, and pepsinogen II; (2) the relatively poor reactivity of anaplastic cancers, both neuroendocrine and non-neuroendocrine, for all markers except MUC1

and, to some extent, CK7; and (3) the generally moderate reactivity of HLR cases with MUC1 and cathepsin E (Fig. 1).

No marker proved diagnostic for any of the nine prognostic subtypes detailed in "Materials and methods." However, (1) the muconodular cancer was extensively reactive for intestinal mucins and CDX2, but not for MUC1, MUC6, PGII, or cathepsin E, (2) the low-grade variant of diffuse desmoplastic cancer was better depicted by CAR5 and LGALS4, and (3) the well-differentiated tubular cancer by CDH17 (Fig. 1).

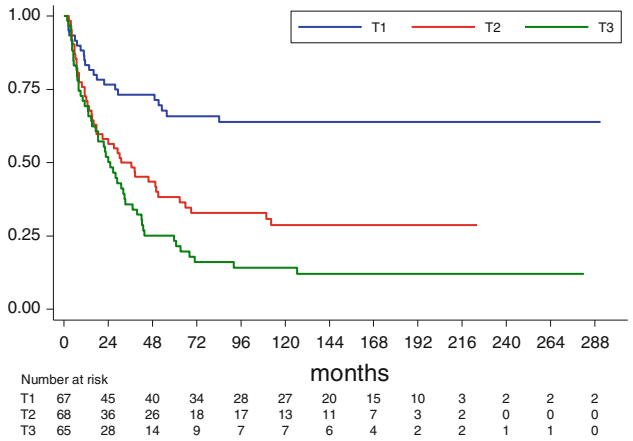

Fig. 2 Kaplan–Meier survival estimate, by tertiles of biomarker burden

No marker proved distinctive of histotype-based grade, although for some markers the expression differences found among the three grades were significant per se (Table 4B). The relatively high expression of CDX2 among grade 1 tumors may fit with the favorable prognostic influence of such marker, whereas their high expression of cathepsin E and MUC1, per se of no or unfavorable prognostic influence, was mainly caused by HLR tumors, showing relatively good survival irrespective of their marker reactivity. Indeed, a total of 45 HLR cases showed clearly better survival at both univariable (HR 0.29, 0.16–0.51, $p < 0.001$) and stage-adjusted (HR 0.45, 0.25–0.82, $p = 0.009$) analysis, compared to the remaining 247 cases.

Prognosis-predictive markers in node-negative and stage I cancers

A systematic analysis of the markers expressed in node-negative neoplasms, showed CDX2 (48/79 positive cases) to be an effective predictor of favorable outcome at both univariable (HR 0.27, 0.11–0.65, $\chi^2 = 8.86$, $p = 0.003$) and stage-inclusive bivariable Cox analysis (HR 0.36, 0.14–0.94, $\chi^2 = 4.32$, $p = 0.036$), whereas PGII (25/93 positive cases) was shown to predict worse survival at both univariable (HR

Table 2 Survival analysis as a function of cancer tissue and cell phenotype

Cancer phenotype	Number of cases	Death rate, 95 % CI	HR	Cox survival analysis		
				95 % CI	*p* value	
					Univariable	With stage
Intestinal	79	6.46, 4.84–8.62	1			
Gastric	57	12.17, 9.02–16.30	1.63	1.07–2.47	0.022	0.012
Mixed	75	12.93, 9.85–16.97	1.60	1.28–2.38	0.020	<0.001
Poor	34	18.02, 12.18–26.67	3.34	1.41–3.75	<0.001	0.003
HLR	16	5.36, 2.56–11.25	0.75	0.32–1.76	0.515	0.631
Anaplastic	15	102.97, 60.98–173.86	7.39	3.60–15.15	<0.001	<0.001

HLR high CD8-positive lymphocyte response

Table 3 Survival analysis of 255 gastric cancers as a function of CDX2/MUC1 expression ratio

	Number	Death		Stage-inclusive Cox analysis			
		Rate	95 % CI	HR	95 % CI	*p* value	
						Univariable	With stage
(a) >20CDX2/ ≤20MUC1	75	6.29	4.47–8.85	1			
(b) >20CDX2/ >20MUC1	76	11.98	8.95–16.05	1.43	0.91–2.24	0.122	0.055
(c) ≤20CDX2/ ≤20MUC1	20	16.46	9.56–28.34	2.01	1.05–3.82	0.034	0.011
(d) ≤20CDX2/ >20MUC1	84	21.02	16.35–27.01	2.31	1.51–3.54	<0.001	<0.001

Table 4 Distribution of phenotypic markers according to gastric cancer histotype (A) and grade (B)

Marker	CDX2	CK20	MUC2	CAR5	CD10	CDH17[a]	LGALS4	MUC5AC	MUC6	PGII	MUC1	CK7	CathE	Lysozyme
(A) Histotype														
HLR	18/41 (44)	11/42 (26)	17/43 (40)	14/45 (31)	6/38 (16)	15/39 (38)	17/39 (44)	18/44 (41)	2/37 (5)	8/44 (18)	36/44 (82)	14/39 (36)	39/45 (87)	20/40 (50)
Cohesive	62/102 (61)	37/101 (37)	37/109 (34)	58/109 (53)	28/99 (28)	50/100 (50)	57/98 (58)	44/110 (40)	29/95 (31)	37/109 (34)	78/105 (74)	52/96 (54)	61/104 (59)	43/96 (45)
Diffuse	38/58 (66)	11/60 (18)	26/72 (36)	48/73 (66)	11/58 (19)	11/57 (19)	32/52 (62)	42/73 (58)	22/67 (33)	29/73 (40)	31/72 (43)	26/62 (42)	44/69 (64)	49/69 (71)
Mucinous	28/37 (76)	11/40 (28)	39/41 (95)	25/42 (60)	9/40 (23)	14/35 (40)	16/37 (43)	27/42 (64)	7/39 (18)	6/42 (14)	15/41 (37)	12/40 (30)	14/41 (34)	8/39 (21)
Anaplastic	6/19 (32)	1/19 (5)	1/17 (6)	1/19 (5)	6/19 (32)	5/19 (26)	1/20 (5)	2/19 (11)	2/11 (18)	2/20 (10)	12/19 (63)	9/19 (47)	6/19 (32)	5/20 (25)
Fisher's exact test, p value	0.005	0.018	<0.001	<0.001	0.428	0.003	<0.001	<0.001	0.007	0.002	<0.001	0.077	<0.001	<0.001
(B) Grade														
1	43/72 (60)	24/79 (30)	42/85 (49)	41/88 (47)	17/75 (23)	29/68 (43)	34/68 (50)	40/87 (46)	10/77 (13)	14/87 (16)	44/85 (52)	27/76 (36)	58/87 (67)	37/78 (47)
2	92/149 (62)	42/146 (29)	61/162 (38)	92/169 (56)	33/143 (23)	57/147 (39)	82/141 (58)	77/164 (47)	46/144 (32)	62/163 (38)	107/159 (67)	72/143 (50)	92/155 (59)	80/149 (54)
3	17/36 (47)	5/37 (14)	17/35 (49)	13/37 (35)	10/36 (28)	9/35 (26)	7/37 (19)	16/37 (43)	6/28 (21)	6/38 (16)	21/37 (57)	14/37 (38)	14/36 (39)	8/37 (22)
Fisher's exact test, p value	0.273	0.122	0.152	0.042	0.800	0.242	<0.001	0.915	0.006	<0.001	0.039	0.082	0.019	0.002

[a] Only membrane expression was considered here; total (membranous + cytoplasmic) expression gave no significant correlation with either histotype or grade

4.01, 1.82–8.81, $\chi^2 = 10.93$, $p = 0.001$) and stage-inclusive analysis (HR 4.82, 2.13–10.93), $\chi^2 = 14.18$, $p < 0.001$). No predictive power was shown, on node-negative cancers, by other markers, MUC1 included.

Survival analysis restricted to stage I tumors showed CDX2 (HR 0.34, 0.14–0.87, $p = 0.024$) and PGII (HR 3.45, 1.52–7.83, $p = 0.003$) to be the best predictors. The results of their combined application to 83 stage I cases are shown in Table 5.

Prediction of lymph node status

Neither CDX2 nor PGII was found to be significantly associated with N status, for which only LGALS4 (as cytoplasmic staining intensity score, OR 0.39, 0.21–0.70, $p = 0.002$) and CDH17 (as membranous staining, OR 0.62, 0.36–1.07, $p = 0.084$) were informative. MUC1 proved effective only after dropping out HLR tumors (OR 2.41, 1.34–4.34, $p = 0.003$; against OR: 1.54, 0.92–2.56, $p = 0.100$ when retaining HLR cases). In fact, HLR histology was found to be per se a strong negative predictor of lymph node metastasis (OR 0.28, 0.15–0.54, $p > 0.001$), thus masking a positive predictive power of MUC1 in non-HLR tumors.

Multivariable analysis

In a multivariable model inclusive of age and sex applied to 255 cases (Table 6) the favorable predictive power of CDX2 proved to be independent of both stage and histotype-based grade, whereas the worsening influence of MUC1 (or, in separate tests, PGII) lost significance. When in the same model the eight biomarkers burden (2 + 3 tertiles) was substituted for both CDX2 and MUC1, a HR of 3.33, 2.03–5.48, $p < 0.001$ was obtained without substantial change of the remaining factors, histotype-based grade included HR 3.44, 1.90–6.21, $p < 0.001$.

Table 5 Cox survival analysis of 83 stage I cancers as a function of CDX2/PGII expression ratio

	Number	HR	95 % CI	*p* value
(a) >20CDX2/ <10PGII	41	1		
(b) ≤20CDX2/ <10PGII	20	4.73	1.13–19.83	0.033
(c) >20CDX2/ ≥10PGII	15	6.78	1.69–27.13	0.007
(d) ≤20CDX2/ ≥10PGII	7	19.53	4.29–88.95	<0.001

Model: $\chi^2(3) = 16.97$, $p < 0.001$; Harrell's $C = 0.768$

Table 6 Multivariable analysis of 255 gastric cancers

	HR	95 % CI	*p* value
Sex	1.17	0.83–1.65	0.361
Age	1.01	1.00–1.03	0.113
Stage			
II	2.97	1.67–5.27	<0.001
III + IV	8.35	4.85–14.37	<0.001
Histotype-based grade, 2 + 3	3.52	2.06–6.00	<0.001
CDX2, >20 %	0.58	0.41–0.83	0.003
MUC1, >20 %	1.20	0.82–1.76	0.357

Model: $\chi^2(7) = 178.2$, $p < 0.001$; Harrell's $C = 0.802$

Discussion

Of the 14 phenotype-related markers tested in this study, only 6 appeared significantly informative of prognosis at univariable Cox analysis of the whole tumor series. Three of these, CDX2, MUC2, and LGALS4, identified a group with improved survival, and 3, PGII, MUC6, and MUC1, identified a group with worse survival. Bivariable analysis confirmed a stage-independent predictive power only for CDX2, MUC1, MUC6, and PGII. However, based on the reasonable hypothesis that no biomarker acts alone as a negative or positive prognostic factor, but it is their collective effect that contributes to the patient's outcome [30], we also analyzed the global influence of 8 potentially informative markers showing $p < 0.1$ at univariable Cox analysis. We found that the resulting "biomarker burden" worked as a strong stage- and histotype-independent factor in assessing patient outcome. This finding, besides representing by itself a proof of principle for a role of phenotypic markers in gastric cancer behavior, allows effective stratification of patients according to their risk.

When normal tissue expression of the markers was considered, a general trend appeared for intestinal phenotype markers to predict improved survival compared to markers normally more (PGII, MUC6, MUC5AC) or less (MUC1) restricted to gastric epithelia, or even to a more pancreatobiliary duct marker such as cytokeratin 7. This behavior was confirmed when cancers with predominantly intestinal phenotype were compared with those with predominantly gastric, mixed (intestinal + gastric or pancreatobiliary), or poorly expressed phenotype. These phenotype-linked prognostic differences confirm and further develop previous results [2, 3, 7, 9, 11, 13, 17, 18]. An important new finding of our study is the identification of two different histological subsets, the anaplastic and the lymphocyte-rich HLR subsets, of cancers with poorly expressed phenotype, showing opposite behavior, in keeping with the known ominous prognosis of the anaplastic histotype [23, 25] and the relatively favorable behavior of the HLR histotype [19, 20, 25, 26]. Indeed, distinction of these two histological subsets allowed more appropriate appreciation of the prognostic value of poor expression of markers.

The reason for the more favorable behavior of cancers with intestinal phenotype remains unknown. An oncosuppressor function of the MUC2 [31] and CDX2 [32, 33] genes has been suggested, based on molecular in vitro and in vivo evidence. The association of MUC1 expression with worse prognosis has been reported in many solid cancers, including gastric cancer [7, 8, 34–36] and attributed to MUC1 protein's direct interaction with the cell membrane, so as to prevent cell adhesion, or with epidermal growth factor (EGF)-related receptors and β-catenin, thus altering cell proliferation and differentiation, or to activation of NFkB signaling, causing an antiapoptotic response [35, 36].

The worse prognostic influence of the mucopeptic cell markers MUC6 and PGII confirms previous findings [9]. Interestingly, the prognostic effect of PGII was more powerful among stage I cancers, whereas MUC1 proved more effective in stage II and III + IV, and CDX2 among all stage groups. These results led us to explore the potential usefulness of a combination of CDX2 and PGII in predicting the behavior of stage I cancers. The model, which appeared to be predictive, might contribute to the perioperative evaluation of early cohesive cancers undergoing (or considered for) endoscopic ablation or limited surgical resection. For this important clinical issue, the assessment of intestinal versus gastric phenotype or of CDH17 status have been proposed [13, 17, 37]. Indeed, in our study, the expression of LGALS4 and CDH17 negatively predicted lymph node metastases, which in contrast were positively correlated with MUC1 expression, but only after dropping HLR cases. In fact, the HLR histotype, which proved by itself, and independently of its MUC1 reactivity, to be a strong negative predictor of lymph node metastasis, would mask the positive predictive power of MUC1 among non-HLR tumors.

Significant differences were found in the distribution of individual markers among tumor histotypes, either expected, such as high mucin marker expression among mucinous and signet ring cancers, or unexpected, such as the overexpression of both cathepsin E and MUC1 in HLR tumors. A role of cathepsin E in antigen processing by professional or acquired immunocompetent cells (including epithelial cells such as intestinal M cells) has been suggested in the past [38, 39], while more recently its expression has been found to be inducible by hematopoietic transcription factors and to be regulated by the HLA class II transactivator [40]. This function in cellular immune response, which fits with the highly enhanced expression of cathepsin E previously reported in *H. pylori*-infected gastric epithelium [41], may point to a role of this cathepsin in eliciting the antitumor T-cell response of HLR cancers.

It seems wise, when analyzing the role of cell phenotype in gastric cancer prognosis, to also consider cancer histotype, whose stage- as well as biomarker-independent predictive power is confirmed by the present multivariable analysis. Special attention should be given to the prognostically informative subtypes among low (grade 1) or high (grade 3) grade groups, known to account for most of the prognostic power inherent to the histotype-based grading system [22, 23]. In particular, HLR tumors, which form the largest group among grade 1 tumors, should be taken into consideration as a potential confounding factor in prognostic studies.

In conclusion, although the prognostic impact of individual tumor cell markers on gastric cancer behavior, with the notable exception of CDX2, seems relatively limited, combination of potentially predictive markers and assessment of tumor cell phenotype provide important prognostic information, especially when integrated with histotype-based tumor grading.

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Conflict of interest The authors have no competing interests to declare.

References

1. Fiocca R, Villani L, Tenti P, Solcia E, Cornaggia M, Frigerio B, et al. Characterization of four main cell types in gastric cancer: foveolar, mucopetic, intestinal columnar and goblet cells. An histopathologic, histochemical and ultrastructural study of "early" and "advanced" tumours. Pathol Res Pract. 1987;182:308–25.
2. Machado JC, Nogueira AMM, Carneiro F, Reis CA, Sobrinho-Simões M. Gastric carcinoma exhibits distinct types of cell differentiation: an immunohistochemical study of trefoil peptides (TTF1 and TTF2) and mucins (MUC1, MUC2, MUC5AC, and MUC6). J Pathol. 2000;190:437–43.
3. Tajima Y, Shimoda T, Nakanishi Y, Yokoyama N, Tanaka T, Shimizu K, et al. Gastric and intestinal phenotypic marker expression in gastric carcinomas and its prognostic significance: immunohistochemical analysis of 136 lesions. Oncology. 2001;61:212–20.
4. Fiocca R, Villani L, Tenti P, Cornaggia M, Finzi G, Riva C, et al. The foveolar cell component of gastric cancer. Hum Pathol. 1990;21:260–70.
5. Bara J, Chastre E, Mahiou J, Singh RL, Forgue-Lafitte ME, Hollande E, et al. Gastric M1 mucin, an early oncofetal marker of colon carcinogenesis, is encoded by the MUC5AC gene. Int J Cancer. 1998;75:767–73.
6. Fiocca R, Villani L, Tenti P, Cornaggia M, Finzi G, Capella C, et al. Widespread expression of intestinal markers in gastric carcinoma: a light and electron microscopy study using BD-5 monoclonal antibody. J Clin Pathol. 1988;41:178–87.
7. Utsunomiya T, Yonezawa S, Sakamoto H, Kitamura H, Hokita S, Aiko T, et al. Expression of MUC1 and MUC2 mucins in gastric carcinomas: its relationship with the prognosis of the patients. Clin Cancer Res. 1998;4:2605–14.

8. Lee HS, Lee HK, Kim HS, Yang HK, Kim YI, Kim WH. MUC1, MUC2, MUC5 and MUC6 expressions in gastric carcinomas. Cancer (Phila). 2001;92:1427–34.
9. Fiocca R, Cornaggia M, Villani L, Capella C, Solcia E, Samloff M. Expression of pepsinogen II in gastric cancer. Its relationship to local invasion and lymph node metastases. Cancer (Phila). 1988;61:956–62.
10. Sato Y, Itoh F, Hinoda Y, Ohe Y, Nakagawa N, Ueda R, et al. Expression of CD10/neutral endopeptidase in normal and malignant tissues of the human stomach and colon. J Gastroenterol. 1996;31:12–7.
11. Mizoshita T, Tsukamoto T, Nakanishi H, Inada K, Ogasawara N, Joh T, et al. Expression of Cdx2 and the phenotype of advanced gastric cancers. Relationship with prognosis. J Cancer Res Clin Oncol. 2003;129:727–34.
12. Kim MA, Lee HS, Yang H-K, Kim WH. Cytokeratin expression profile in gastric carcinomas. Hum Pathol. 2004;5:576–81.
13. Park SS, Kang SH, Park JM, Kim JH, Oh SC, Lee JH, et al. Expression of liver-intestine cadherin and its correlation with lymph node metastasis in gastric cancer: can it predict N stage preoperatively? Ann Surg Oncol. 2007;14:94–9.
14. Laurén P. The two histological main types of gastric carcinoma. Diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49.
15. Chen X, Leung SY, Yuen ST, Chu KM, Ji J, Li R, et al. Variation in gene expression patterns in human gastric cancers. Mol Biol Cell. 2003;14:3208–15.
16. Tan IB, Ivanova T, Lim KH, Ong CW, Deng N, Lee J, et al. Intrinsic subtypes of gastric cancers, based on gene expression pattern, predict survival and respond differently to chemotherapy. Gastroenterology. 2011;141:476–85.
17. Koseki K, Takizawa T, Koike M, Ito M, Nihei Z, Sugihara K. Distinction of differentiated type early gastric carcinoma with gastric type mucin expression. Cancer (Phila). 2000;89:724–32.
18. Han HS, Lee SY, Lee KY, Hong SN, Kim JH, Sung IK, et al. Unclassified mucin phenotype of gastric adenocarcinoma exhibits the highest invasiveness. J Gastroenterol Hepatol. 2008;24:658–66.
19. Minamoto T, Mai M, Watanabe K, Ooi A, Kitamura T, Takahashi Y, et al. Medullary carcinoma with lymphocytic infiltration of the stomach. Clinicopathologic study of 27 cases and immunohistochemical analysis of the subpopulations of infiltrating lymphocytes in the tumor. Cancer (Phila). 1990;66(5):945–52.
20. Grogg KL, Lohse CM, Pankratz VS, Halling KC, Smyrk TC. Lymphocyte-rich gastric cancer: associations with Epstein–Barr virus, microsatellite instability, histology, and survival. Mod Pathol. 2003;16:641–51.
21. Endoh Y, Tamura G, Motoyama T, Ajioka Y, Watanabe H. Well differentiated adenocarcinoma mimicking complete-type intestinal metaplasia in the stomach. Hum Pathol. 1999;30:826–32.
22. Solcia E, Luinetti O, Tava F, Klersy C, Grillo F, Pandolfo N, et al. Identification of a lower grade muconodular subtype of gastric mucinous cancer. Virchows Arch. 2004;445:572–9.
23. Chiaravalli AM, Klersy C, Tava F, Manca R, Fiocca R, Capella C, et al. Lower- and higher-grade subtypes of diffuse gastric cancer. Hum Pathol. 2009;40:1591–9.
24. Rossi E, Klersy C, Manca R, Zuffardi O, Solcia E. Correlation between genomic alterations assessed by array comparative genomic hybridization, prognostically informative histologic subtype, stage, and patient survival in gastric cancer. Hum Pathol. 2011;42:1937–45.
25. Solcia E, Klersy C, Mastracci L, Alberizzi P, Candusso ME, Diegoli M, et al. A combined histologic and molecular approach identifies three groups of gastric cancer with different prognosis. Virchows Arch. 2009;455:197–211.
26. Chiaravalli A, Klersy C, Vanoli A, Ferretti A, Capella C, Solcia E. Histotype-based prognostic classification of gastric cancer. World J Gastroenterol. 2012;18(9):896–904.
27. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma: 2nd English edition. Gastric Cancer. 1998;1:10–24.
28. Lauwers GY, Carneiro F, Graham DY, Curado MP, Franceschi S, Montgomery E et al. Gastric carcinoma. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, editors. WHO classification of tumours of the digestive system. Lyon: IARC; 2010.
29. Rindi G, Azzoni C, La Rosa S, Klersy C, Paolotti D, Rappel S, et al. ECL cell tumour and poorly differentiated endocrine carcinoma of the stomach: prognostic evaluation by pathological analysis. Gastroenterology. 1999;116:532–42.
30. Zethelius B, Berglund L, Sundstrom J, Ingelsson E, Basu S, Larsson A, et al. Use of multiple biomarkers to improve the prediction of death from cardiovascular causes. N Engl J Med. 2008;358:2107–16.
31. Velcich A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S, et al. Colorectal cancer in mice genetically deficient in the mucin MUC2. Science. 2002;295(5560):1726–9.
32. Bai YQ, Miyake S, Iwai T, Yuasa Y. CDX2, a homeobox transcription factor, upregulates transcription of the p21/WAF1(CIP1 gene). Oncogene. 2003;22:5998–6005.
33. Guo R-J, Funakoshi S, Lee HH, Kong J, Lynch JP. The intestine-specific transcription factor Cdx2 inhibits β-catenin-TCF protein complex. Carcinogenesis (Oxf). 2010;31:159–66.
34. Tajima Y, Yamazaki K, Nishino N, Morohara K, Yamazaki T, Kaetsu T, et al. Gastric and intestinal phenotypic marker expression in gastric carcinomas and recurrence pattern after surgery-immunohistochemical analysis of 213 lesions. Br J Cancer. 2004;91:1342–8.
35. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control on the cell surface. Cancer (Phila). 2004;4:45–59.
36. Ahmad R, Raina D, Trivedi V, Ren J, Rajabi H, Kharbanda S, et al. MUC1 oncoprotein activates the IkappaB kinase beta complex and constitutive NF-kappaB signalling. Nat Cell Biol. 2007;9:1419–27.
37. Lee HJ, Nam KT, Park HS, Kim MA, Lafleur BJ, Aburatani H, et al. Gene expression profiling of metaplastic lineages identifies CDH17 as a prognostic marker in early stage gastric cancer. Gastroenterology. 2010;139:213–25.
38. Bennett K, Levine T, Ellis JS, Peanasky RJ, Samloff IM, Kay J, et al. Antigen processing for presentation by class II major histocompatibility complex requires cleavage by cathepsin E. Eur J Immunol. 1992;22:1519–24.
39. Finzi G, Cornaggia M, Capella C, Fiocca R, Bosi F, Solcia E, et al. Cathepsin E in follicole-associated epithelium of intestine and tonsils. Its localization to M cells and possible role in antigen processing. Histochemistry. 1993;99:201–11.
40. Yee CS, Yao Y, Li P, Klemsz MJ, Blum JS, Chang C-H. Cathepsin E: a novel target for regulation by class II transactivator. J Immunol. 2004;172:5528–34.
41. Fiocca R, Luinetti O, Villani L, Chiaravalli AM, Capella C, Solcia E. Epithelial cytotoxicity, immune response, and inflammatory components of *Helicobacter pylori* gastritis. Scand J Gastroenterol. 1994;29(suppl 205):11–21.