Gastric Cancer (2014) 17:442–449
DOI 10.1007/s10120-013-0315-1

ORIGINAL ARTICLE

High-throughput mutation profiling identifies novel molecular dysregulation in high-grade intraepithelial neoplasia and early gastric cancers

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Received: 29 July 2013 / Accepted: 4 November 2013 / Published online: 23 November 2013

Abstract
Background There is still no widely accepted molecular marker available to distinguish between gastric high-grade intraepithelial neoplasia (HG-IEN) and invasive early gastric cancer (EGC).
Methods HG-IEN and EGC lesions coexisting in the same patient were manually microdissected from a series of 15 gastrectomies for EGC; 40 ng DNA was used for multiplex PCR amplification using the Ion AmpliSeq Cancer Panel, which explores the mutational status of hotspot regions in 50 cancer-associated genes.
Results Of the 15 EGCs, 12 presented at least one somatic mutation among the 50 investigated genes, and 6 of these showed multiple driver gene somatic mutations. *TP53* mutations were observed in 9 cases; *APC* mutations were identified in 3 cases; and *ATM* and *STK11* were mutated in 2 cases. Seven HG-IEN lesions shared an identical mutational profile with the EGC from the same patient; 13 mutations observed in *APC*, *ATM*, *FGFR3*, *PIK3CA*, *RB1*, *STK11*, and *TP53* genes were shared by both HG-IEN and ECG lesions. *CDKN2A*, *IDH2*, *MET*, and *RET* mutations were observed only in EGC. *TP53* deregulation was further investigated in an independent series of 75 biopsies corresponding to all the phenotypic lesions occurring in the EGC carcinogenetic cascade. p53 nuclear immunoreaction progressively increased along with the dedifferentiation of the lesions ($P < 0.001$). Overall, 18 of 20 p53-positive lesions showed a *TP53* mutated gene.
Discussion Our results support the molecular similarity between HG-IEN and EGC and suggest a relevant role for *TP53* in the progression to the invasive phenotype and the use of immunohistochemistry as a surrogate to detect *TP53* gene mutations.

Keywords Gastric carcinogenesis · Intraepithelial neoplasia · Early gastric cancer · Next-generation sequencing · Biomarkers

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Introduction

Gastric adenocarcinoma is the fourth most common type of cancer and the second leading cause of cancer deaths worldwide [1]. Intestinal-type adenocarcinoma is the final outcome of long-standing gastritis caused primarily by *Helicobacter pylori* infection [2]. Chronic gastritis leads to the replacement of the native epithelium with intestinal metaplasia, the 'carcinogenic field' in which initially dysplastic lesions and subsequently adenocarcinomas may develop [3].

In spite of well-established knowledge about morphological lesions occurring in the gastric carcinogenetic cascade, molecular typing of the precancerous changes in gastric mucosa remains elusive [4]. In fact, no molecular marker is yet available in clinical practice to enable the distinction between high-grade intraepithelial neoplasia (HG-IEN; formerly known as high-grade dysplasia) and early gastric cancer (EGC) invasive lesions [4, 5]. Moreover, data from currently available literature pinpoint that use of dysplasia alone as a biomarker has severe limitations from both an endoscopic and a histological aspect, which affects the efficiency of planned screening/surveillance programs [6, 7]. Thus, new molecular biomarkers are needed to adequately stratify patients according to their cancer risk.

Efforts to establish a direct molecular link between histological phenotypes and risk of malignancy have, to date, been limited by the availability of only partially degraded DNA from formalin-fixed and paraffin-embedded (FFPE) tissue. Massive parallel sequencing, also known as next-generation sequencing (NGS) or deep sequencing, is the most sensitive approach to index multiple genes even with only a limited amount of DNA from different sources including FFPE tissues [8, 9]. NGS can simultaneously investigate multiple potential molecular targets and represents a potent diagnostic complement to histopathological and immunophenotypic diagnosis.

In this setting, we used the Ion Torrent Personal Genome Machine (PGM) genotyping platform to screen a series of matched noninvasive and early invasive intestinal-type gastric neoplastic lesions for oncogenic hotspot mutations in 50 cancer-related genes.

Materials and methods

Cases

A retrospective series of 15 sporadic well-differentiated intestinal-type EGC (10 men; age 57.6 ± 9.5 years) and coexisting HG-IEN lesions were subjected to the mutational study (Table 1). The cases were retrieved from the archives of the Department of Pathology at the University of Verona. All tumors showed a low (G1) or moderate (G2) degree of differentiation. All patients had been surgically treated at the same institution (First Surgical Division of the University of Verona) and had not received neoadjuvant chemotherapy. The gross features of the tumor were obtained from both the gross description of the specimen as recorded at the time of surgery and from the original histopathology report. All EGCs were located in stomachs that had stage III–IV atrophic gastritis, according to Operative Link on Gastritis Assessment (OLGA) classification [3, 10], and presented a HG-IEN lesion distinct from the EGC lesion. The extension of gastric mucosa atrophy and intestinal metaplasia was assessed histologically from the original hematoxylin and eosin (H&E) slides.

Table 1 Clinicopathological characteristics of the considered series

#	Sex	Age (years)	Site	Macroscopic growth pattern[a]	Size (mm)	Grading
1	M	68.5	Incisura angularis	Fungating	12	G1
2	M	54.7	Incisura angularis	Fungating	10	G2
3	M	43.8	Incisura angularis	Fungating	11	G1
4	M	62.3	Antrum	Polypoid	29	G1
5	M	48.3	Antrum	Fungating	18	G1
6	M	54.2	Incisura angularis	Fungating	13	G2
7	M	59.5	Incisura angularis	Fungating	14	G1
8	M	65.1	Antrum	Fungating	9	G1
9	M	60.5	Incisura angularis	Polypoid	20	G2
10	M	72.2	Antrum	Fungating	14	G1
11	F	70.8	Incisura angularis	Fungating	13	G1
12	F	54.2	Incisura angularis	Fungating	17	G1
13	F	53.9	Antrum	Polypoid	24	G1
14	F	40.2	Antrum	Polypoid	18	G2
15	F	55.2	Antrum	Fungating	18	G1

[a] According to the Borrmann classification [10]

A further series of 75 endoscopic biopsy samples from the same number of patients was used for the immunohistochemical study of p53. All biopsies were taken from the distal gastric mucosa, i.e., antrum and/or incisura angularis, and included (1) 15 biopsies of normal antral mucosa, obtained from dyspeptic patients; (2) 15 biopsies of antral mucosa with extensive intestinal metaplasia, obtained from cases of atrophic gastritis in OLGA stages III–IV [3, 10]; (3) 15 cases of low-grade IEN (LG-IEN); (4) 15 cases of HG-IEN; and (5) 15 cases of sporadic well- or moderately differentiated intestinal-type antral EGC (all stage 1 cases).

Original H&E slides were jointly reevaluated and reclassified according to WHO 2010 criteria [11] by two GI pathologists (M.F., C.L.), blinded to all clinical and pathological information; two additional expert pathologists (P.C., A.T.) evaluated cases of discordant classification. Intraepithelial neoplasia (IEN) was defined by the presence of epithelial neoplastic proliferation without evidence of invasive growth. IEN lesions were then further stratified as low grade or high grade according to the severity of

cellular and architectural atypia [11]. ECG were defined as invasive adenocarcinoma limited to the mucosa, or the mucosa and submucosa, regardless of nodal status. The study was approved by the local ethics committee of the Integrated University Hospital Trust of Verona (AIRC No. 6421/2008).

Enrichment for neoplastic cellularity, DNA extraction, and qualification

The HG-IEN and EGC lesions were manually microdissected from five to ten consecutive 4-μm-thick sections of FFPE specimens to ensure that each tumor sample contained at least 60 % neoplastic cells. In all cases, the two considered lesions, HG-IEN and EGC, were separated by the presence of normal, metaplastic, or LG-IEN mucosa (Fig. 1). Normal gastric tissue was used to determine the presence of germline variants and somatic mutations. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Milan, Italy). Purified DNA was quantified and its quality assessed using NanoDrop (Invitrogen Life Technologies, Milan, Italy) and Qubit (Invitrogen Life Technologies) platforms [12]. Suitability of DNA derived from FFPE specimens for PCR downstream application was further evaluated by polymerase chain reaction (PCR) analysis through BIOMED 2 PCR multiplex protocol [13] and the PCR products were analyzed by DNA 1000 Assay (Invitrogen Life Technologies) on the Agilent 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies, Santa Clara, CA, USA).

Deep sequencing of multiplex PCR amplicons

For multiplex PCR amplification, 40 ng DNA was used, using the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) that explores selected regions of the following 50 cancer-associated genes (in alphabetical order): *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HNF1A, HRAS, JAK2, JAK3, IDH1, IDH2, KDR/VEGFR2, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL.*

Emulsion PCR was performed with the OneTouch OT2 system (Life Technologies). The quality of the obtained library was evaluated by High Sensitivity Assay on the Agilent 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies). Sequencing was run on the Ion Torrent PGM (Life Technologies) loaded with a 316 chip. Data analysis, including alignment to the hg19 human reference genome and variant calling, utilized the Torrent Suite Software ver. 3.6 (Life Technologies). Filtered variants were annotated using the SnpEff software ver. 3.1 (alignments visually verified with the Integrative Genomics Viewer; IGV ver. 2.1, Broad Institute).

TP53 mutational status and p53 immunohistochemical staining

TP53 gene mutations detected by deep sequencing were confirmed by PCR amplification of appropriate fragments

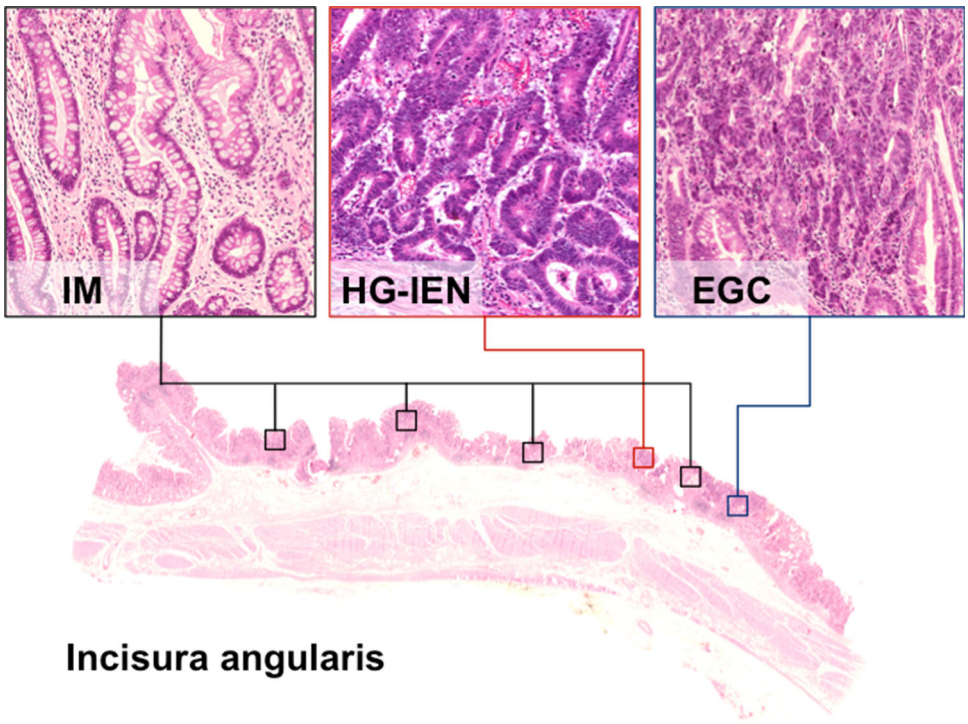

Fig. 1 Case no. 3. The specimen contains diverse lesions shown at higher magnification (*squares*): *HG-IEN* high-grade intraepithelial neoplasia, *IM* intestinal metaplasia, *EGC* early gastric cancer. Hematoxylin and eosin (H&E) ×4, ×10

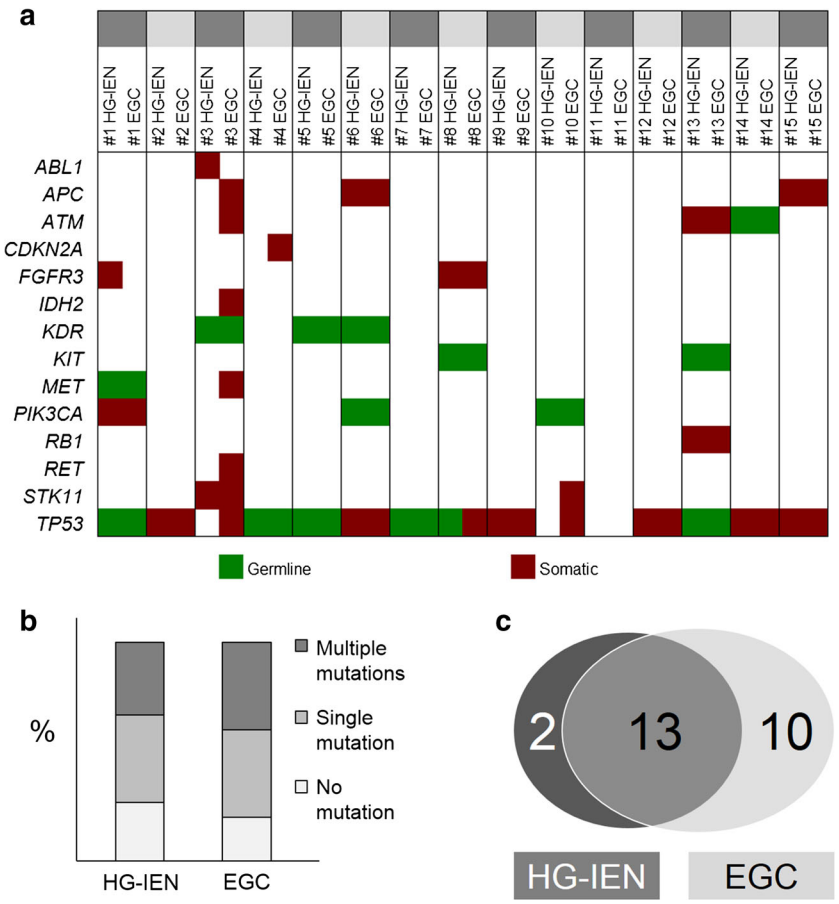

Fig. 2 Significantly mutated genes in matched noninvasive and early invasive gastric lesions as identified by Ion Torrent sequencing. **a** Mutations in cancer-associated genes detected by Ion AmpliSeq Cancer panel. *Columns* denote the matched lesions in each patient; *rows* genes. *Green* and *red* represent germline nonpathological variants and pathological somatic mutations, respectively. **b** Distribution of mutations among the two classes of lesions. **c** Thirteen somatic mutations were seen in both high-grade intraepithelial neoplasia (HG-IEN) and early gastric cancer (EGC) samples, whereas 2 and 10 mutations were observed in only HG-IEN or EGC samples, respectively

and conventional Sanger sequencing. PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter) and labeled with Big Dye Terminator v3.1 (Applied Biosystems). Agencourt CleanSEQ magnetic beads (Beckman Coulter) were used for postlabeling DNA fragment purification, and sequence analysis was performed on an Applied Biosystems 3130xl Genetic Analyzer.

Immunohistochemical staining for p53 using DO-1 antibody (Immunotech; prediluted) were obtained on 4-µm-thick FFPE sections using an automated instrument (BondmaX, Menarini). Sections were lightly counterstained with hematoxylin. Appropriate positive and negative controls were run concurrently. Slides were scored by two pathologists (M.F., C.L.) and a consensus score was reached.

Results

Prevalence of driver genes mutations in gastric HG-IEN and EGC

The 40 ng DNA obtained from the 15 HG-IEN and EGC lesions coexisting in the same patient was subjected to deep sequencing of mutational hotspots of 50 cancer-associated genes. In all 30 samples, an adequate library for subsequent deep sequencing was obtained. A mean

Table 2 Somatic mutations found in matched noninvasive and early invasive gastric neoplastic lesions sequenced for 50 cancer-related genes

Gene	Frequency in HG-IEN	Frequency in EGC	Mutations
ABL1	1/15	0/15	G269R
APC	2/15	3/15	Q1444X, R1450X, S1503X
ATM	1/15	2/15	V410A, A2726V
CDKN2A	0/15	1/15	H83Y
FGFR3	2/15	1/15	F384L, N644D
IDH2	0/15	1/15	G145R
MET	0/15	1/15	F831S
PIK3CA	1/15	1/15	A1066T
RB1	1/15	1/15	I752N
RET	0/15	1/15	E762G
STK11	1/15	2/15	G58S, F354L
TP53	6/15	9/15	A161T, R175H, R196X, R213Q, S241F, F270L, R272C, R282W, R337C

HG-IEN high-grade intraepithelial neoplasia, *EGC* early gastric cancer

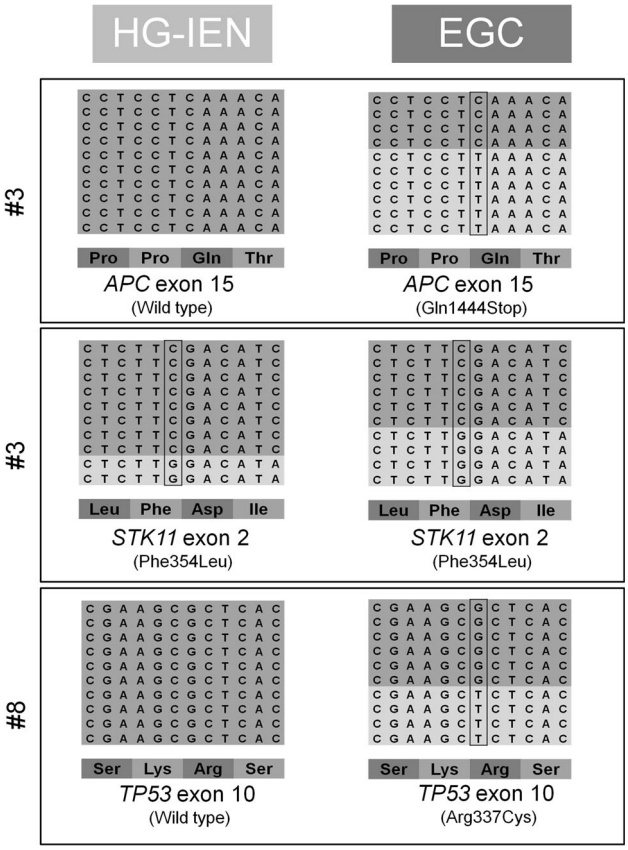

Fig. 3 Examples of mutations detected in matched HG-IEN and EGC lesions. Mutations of *APC* and *STK11* in case 3 and *TP53* in case 8. *Upper panel:* codons codifying for amino acids 1442–1445 of the *APC* gene; the substitution at the first base of codon 1444 causing a stop codon (Q1444*) is found only in the EGC specimen. *Middle panel:* codons codifying for amino acids 353–356 of the *STK11* gene; a F354L mutation was observed in both high-grade intraepithelial neoplasia (HG-IEN) and early gastric cancer (EGC) lesions. *Lower panel:* codons codifying for amino acids 335–338 of the *TP53* gene; a R337C mutation was observed in the EGC specimen only. *Panels* are representations of the reads aligned to the reference genome as provided by the Integrative Genomics Viewer (IGV ver. 2.1, Broad Institute) software

100× coverage of 98.2 % with a mean read length of 105 bp was achieved.

A total of 38 somatic mutations were observed among the 50 cancer-related genes (Fig. 2a). In 22 of 30 (73 %) samples, at least one somatic mutation was detected (Fig. 2b); 11 of 30 lesions (37 %) were found to have multiple driver gene somatic mutations (Fig. 2b).

TP53 gene somatic mutations were the most frequent genetic alterations observed in the series, occurring in 6 of 15 (40 %) HG-IEN and 9 of 15 (60 %) EGC, respectively. Somatic mutations in other genes were detected at lower frequencies in EGC: *APC* (3/15, 20 %); *ATM, STK11* (2/15, 13 %); *PIK3CA*, *RB1*, *CDKN2A*, *FGFR3, IDH2*, *MET*, *RET* (1/15, 7 %) (Table 2).

Germline nonpathological variants in at least one of the following genes were also detected in 20 of 30 samples: *ATM*, *KDR*, *KIT*, *MET*, *PIK3CA*, and *TP53* (Fig. 2a).

Mutation in matched HG-IEN and EGC lesions

Seven cases presented a similar mutational profile in both the HG-IEN and EGC lesions of the same patient (#2, #6, #9, #12, #13, #14, #15) (Fig. 2a) whereas in five cases the HG-IEN and EGC lesions had different molecular profiles (#1, #3, #4, #8, #10). Three cases (#5, #7, #11) showed no mutation in any of the tested 50 cancer-related genes in both lesions. Thirteen mutations observed in the *APC*, *ATM*, *FGFR3*, *PIK3CA*, *RB1*, *STK11*, and *TP53* genes were shared by both HG-IEN and EGC samples of the same patient. Somatic point mutations in the *APC*, *ATM*, *CDKN2A*, *IDH2*, *MET*, *RET*, *STK11*, and *TP53* genes were found exclusively in the EGC lesions of four cases (#3, #4, #8, #10) (Fig. 2c). Examples of mutational results from paired samples are shown in Fig. 3 and Supplementary Table 1.

Correspondence between *TP53* gene mutations and p53 immunohistochemical positivity

DO-1 antibody recognizes a fixative-resistant epitope on the N-terminal amino acids 37 and 45. DO-1 reacts with both wild-type and mutant forms of p53. However, the normal protein has a very short half-life. Only the mutant form, whose half-life is longer, can be immunostained.

Eight of the nine EGCs presenting *TP53* somatic mutations showed a strong p53 nuclear immunostaining in more than 50 % of the neoplastic cells, as was also observed in matched HG-IENs. In the negative case (case #6), the immunohistochemical negativity may be explained by the fact that the R196Stop mutation likely prevents p53 stabilization, as reported in the HuT-78 T-lymphoma cell line harboring the same homozygous nonsense TP53 R196Stop mutation [14].

To further test p53 IHC feasibility as a marker of *TP53* mutational status, we considered an independent series of 75 biopsy specimens corresponding to all the phenotypic lesions occurring in the intestinal-type gastric carcinogenetic cascade. P53 nuclear immunoreaction progressively increased along with the dedifferentiation of the lesions considered (Kruskal–Wallis: $P < 0.001$; Fig. 4). No p53 immunoreaction was seen in normal gastric epithelia, whereas low p53 scores (i.e., < 50 % of positive epithelia) were seen in intestinal metaplastic epithelia. Immunohistochemical p53 nuclear accumulation was observed in 3 of 15 (20 %) LG-IEN, 6 of 15 (40 %) HG-IEN, and 11 of 15 (73 %) EGC samples.

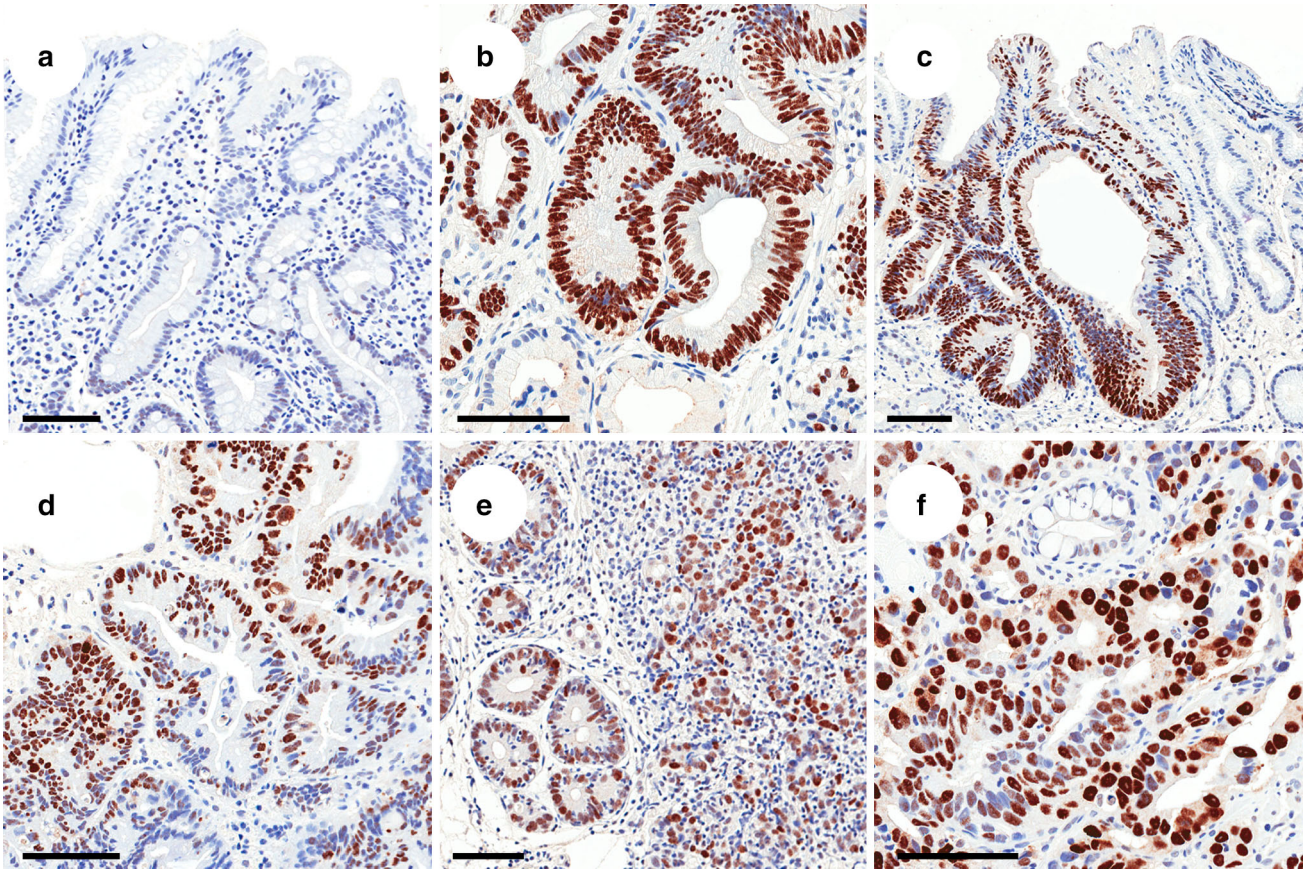

Fig. 4 p53 is dysregulated in gastric carcinogenesis: representative images of p53 immunohistochemical expression. **a** p53 negative intestinal metaplasia. **b**, **c** Positive low-grade intraepithelial neoplasia (LG-IEN) lesions, coexisting with negative normal epithelia. **d** Heterogeneous immunoreactions for p53 in HG-IEN. **e** Diffuse p53 immunoreaction in noninvasive (*left*) and invasive (*right*) components. **f** A p53-positive, well-differentiated adenocarcinoma. *Bars* 100 μm

Discussion

In spite of our well-established understanding of the natural phenotypic history occurring in the progression from native epithelia to invasive intestinal-type carcinomas in the gastric mucosa, the differential diagnosis between high-grade IEN lesions and early invasive gastric adenocarcinomas may be significantly affected by both endoscopic and histological limitations for cases in which only minute biopsy specimens are available. New molecular biomarkers are thus warranted to adequately stratify patients according to their risk of developing invasive cancer [4].

The molecular background of intestinal-type gastric adenocarcinoma has yet to be deciphered [4, 15]. Previous data on molecular profiles of gastric precancerous lesions are based on the analysis of a very limited number of genes and cases [4]. A recent whole-exome sequencing study using DNA from frozen tissues identified recurrent mutations in cell adhesion and chromatin remodeling genes [16]. In this series, frequently mutated genes included *TP53*, *PIK3CA*, and *ARID1A*. Such an approach is not, however, applicable for the study of premalignant lesions. In fact, the adequate histopathological classification and subsequent microdissection needed to isolate these minute lesions imperatively requires the use of FFPE tissue, which is currently insufficient for routine application of whole-genome or whole-exome sequencing [4]. Moreover, most of the limited amount of material obtained from upper GI endoscopy is required for histopathological characterization of the specimen. As a result, there is still no molecular marker used in clinical practice to distinguish HG-IEN from early invasive neoplasia [17–20].

In this study, we explored the mutational status of 50 cancer-related genes using a targeted NGS approach on a series of 15 early gastric adenocarcinomas compared to their matched intestinal-type HG-IEN lesion; this is the first series of premalignant and early invasive gastric mucosa lesions tested through NGS technology. Moreover, the histologically based selection of the cases allowed (1) inter-pathologist reclassification of the selected lesions, to exclude major HG-IEN misdiagnoses; and (2) the inclusion of minute well-differentiated early invasive lesions.

The 15 EGC harbored frequent somatic mutations in *TP53* and *APC* as already reported [4]. *TP53* mutations were identified in 9 of 15 (60 %) of EGC, and most of them involved exons 5–8 and 10. The previously reported incidence of *TP53* mutations in gastric cancer series ranges from 3 % to 65 % [4, 21]. *APC* mutations were found in 3 of 15 (20 %) of our cases; these are relatively rare in extracolonic cancers but have been reported in 5–10 % of gastric cancer series [4, 21].

Two somatic mutations were identified in the *STK11* and *ATM* genes. A germline *STK11* mutated gene is associated with the Peutz–Jeghers syndrome and Peutz–Jeghers-associated gastric hyperplastic polyps [22]. A recent study on a large series of sporadic GC showed that *STK11* was one of the low-frequency mutated genes with a 2 % prevalence [21]. Dysregulation of the ataxia telangiectasia mutated (*ATM*) gene has been associated with microsatellite instability (MSI) and is an independent negative prognostic factor in gastric cancer patients after surgery [23].

In our study, only one *PIK3CA* somatic mutation was observed, which is a slightly lower incidence than previous reports [21]. This finding could be explained by the lower prevalence of *PIK3CA* mutations observed in intestinal-type cancers [24]. *PIK3CA* germline nonpathological variants were present in two cases.

Here we describe for the first time a comprehensive molecular profiling of HG-IEN lesions. Most of these showed a similar mutational status to their invasive counterpart. In particular, 6 of 9 *TP53*, 2 of 3 *APC*, 1 of 2 *ATM* and *STK11*, and 1 of 1 *PIK3CA*, *RB1*, and *FGFR3* mutations observed in adenocarcinomas were shared with their noninvasive lesions, supporting the consideration of a relatively early role for these genes in gastric mucosa transformation. On the other hand, single *CDKN2A*, *IDH2*, *MET*, and *RET* somatic mutations were observed only in the invasive counterpart of matched samples. *IDH2* mutations have never been described in gastric cancers and are rare in the gastrointestinal tract, with the exception of intrahepatic cholangiocarcinomas [25]. The diagnostic impact of *CDKN2A*, *MET*, and *RET* in discriminating among preinvasive and early invasive lesions should be tested in a larger prospective series of HG-IEN, whereas the germline variants in *ATM*, *KDR*, *KIT*, *PIK3CA*, and *TP53* that are shared by both HG-IEN and EGC lesions should be evaluated for their potential to identify predisposition to gastric cancer.

The comparable mutational profiles of matched HG-IENs and EGCs further support the malignant potential of HG-IEN lesions. From a classification aspect, these molecular findings support the Japanese definition of HG-IEN as "non-invasive intramucosal carcinoma." On the other hand, further studies using NGS technology should consider LG-IEN lesions to assess the molecular carcinogenetic cascade of intestinal-type gastric cancer to define a new and widely accepted classification system.

As *TP53* was the most common mutated gene, we further explored *TP53* dysregulation in gastric carcinogenesis using immunohistochemical analysis as a surrogate marker for *TP53* gene alterations. Among the 50 tested genes, *TP53* was the best candidate as a diagnostic and prognostic biomarker of gastric epithelia transformation from dysplasia to early invasive cancer. Previous studies demonstrated loss of heterozygosis at the *TP53* locus in 14 % of a series of gastric intestinal metaplasia and in 22 % of gastric dysplastic lesions [17]. In our series, 6 of 9 EGC-coexisting HG-IEN lesions were *TP53* mutated, and p53 overexpression increased with the increasing severity of the precancerous lesion in an independent set of 75 biopsy specimens. Moreover, p53 immunohistochemistry was consistent with mutational status, which is critical in a clinical setting because of the feasibility of immunohistochemical staining on biopsy material. In the discovery set of 15 samples, 8 of 9 *TP53* mutated cases showed a strong nuclear p53 immunoreaction. Of interest, the p53 negative sample was the R196stop mutation, which also suggests the concurrent loss of the complementary allele.

Overall, these data pointed to a late involvement of p53 dysregulation in the intestinal-type gastric oncogenic process, and support the clinical use of p53 immunohistochemical evaluation, as a surrogate of TP53 somatic mutation, in HG-IEN to identify cancer-prone cases of gastric dysplasia that require closer follow up or more aggressive therapy. The known fact that not all TP53 mutated cases are p53 immunohistochemically positive, because of lack of expression of the mutated protein and loss of the normal allele by loss of heterozygosity, would suggest the necessity to first perform the immunohistochemical staining and, if negative, to test *TP53* mutational status in a clinical setting.

Our high-throughput mutation profiling identifies novel molecular dysregulation in HG-IEN and EGCs. Our results support the molecular similarity between HG-IEN and EGC and suggest a relevant role for *TP53* in the progression to the invasive phenotype whose alteration may be identified by immunohistochemical analysis. The compatibility of NGS technology with formalin-fixed and paraffin-embedded tissues represents a strong driver for the future identification of molecular biomarkers of cancer progression in gastric mucosa. This achievement will lead to the development of new molecular-based secondary prevention approaches in the selection of patients at high risk of gastric cancer.

Acknowledgments We thank Rita T. Lawlor for helpful discussion; Associazione Italiana Ricerca Cancro (AIRC Grants Nos. 12182 and

6421); Italian Cancer Genome Project (FIRB RBAP10AHJB). The funding agencies had no role in the collection, analysis, and interpretation of data and in the writing of the manuscript.

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

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