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Oncogenic mutations in gastric cancer with microsatellite instability

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ABSTRACT

Aim: Mitogen-activated protein kinase (*MAPK*) cascade and phosphatidylinositol 3-kinase (*PI3K*) survival pathways are frequently activated in the progression of gastrointestinal malignancies. In this study, we aimed to determine the frequency of gene mutations in members of these pathways – Epithelial Growth Factor Receptor (*EGFR*), *KRAS*, *BRAF*, *PIK3CA* and *MLK3* in a series of 63 gastric carcinomas with high levels of microsatellite instability (MSI).

Methods: Gene mutation analysis was performed by PCR amplification followed by direct sequencing. In selected tumour cases, *EGFR* expression was evaluated by immunohistochemistry. Association studies between molecular data and clinicopathologic characteristics were performed.

Results: Mutations in *EGFR* (3′-untranslated region [UTR] polyA repeat), *KRAS*, *PIK3CA* and *MLK3* genes occurred in 30 (47.6%), 11 (17.5%), 9 (14.3%) and 2 (3.2%) of the MSI gastric cancer (GC) cases, respectively. No *BRAF* or *EGFR* hotspot mutations were identified. Overall, mutations in at least one of these genes were found in 55.6% (35/63) of gastric carcinomas. From those mutant cases 40.0% (14/35) of them had concomitant gene mutations, always involving *EGFR* polyA deletions. Interestingly, we observed significant associations between oncogenic mutations and female gender ($p = 0.046$) old age of diagnosis ($p = 0.001$) and intestinal subtype ($p = 0.043$).

Conclusion: Our results show that MSI gastric carcinoma frequently shows activation of *EGFR-MAPK* and *PI3K* pathways. Within all alterations found, deletions of the A13 repeats of *EGFR* were common, suggesting this molecular event as an important biomarker for stratification of GC patients for treatment with *EGFR* inhibitors.

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1. Introduction

EGFR is a transmembrane protein that homo- or heterodimerizes with other *EGFR* family members at the cell membrane.[1] Receptor dimerisation causes activation of the intrinsic cytoplasmic kinase domain, resulting in the phosphorylation of several tyrosine residues.[2] The active *EGFR* stimulates the *MAPK* cascade and *PI3K* survival pathways.[1] In some neoplasias, such as in non-small cell lung cancer, it has been demonstrated that patients with tumours harbouring structural alterations on the *EGFR* kinase domain could benefit from the pharmacological treatment with *EGFR* inhibitors.[3] However, it is well known that in lung and also colon cancer, the clinical response to *EGFR* inhibitors depends on the tumour genetic profile. Moreover, it has been clearly demonstrated that patients with metastatic colon cancer harbouring mutations in *EGFR* downstream molecules, namely in *KRAS* or *BRAF* genes, are resistant to *EGFR* inhibitors, specifically to the anti-*EGFR* monoclonal antibody cetuximab.[4–7]

Recently, Yuan[14] found a novel mechanism for *EGFR* activation occurring in colon carcinomas with MSI phenotype; mutations in an A13 repeat located at the 3′ (UTR) of gene. Further, mutations in this region of *EGFR* were found to be associated with *EGFR* overexpression.[14]

In GC, and in particular in the MSI subset, data on *EGFR* alterations as well as mutations on its downstream targets, namely those belonging to the *MAPK* and *PI3K* pathways, are very limited. Some authors reported that *EGFR* is over-expressed in a maximum frequency of 38% of GC[8–10] and very few cases were reported to harbour gene structural alterations like gene amplification or mutations.[11–13]

In GC, the *KRAS* gene mutation frequency varies between 3 to 8% and whenever present, *KRAS* mutations normally cluster in the MSI subset (~30% of MSI cases).[15–19] In contrast, others and we found that *BRAF* mutations rarely occur in this type of epithelial cancer.[15,20–23] We have previously reported mutations in *PIK3CA* gene in MSI GC[18] and recently, our group have also reported mutations in the *MLK3* gene, which is a component of the multiprotein *BRAF/RAF1* complex, in MSI gastric and colorectal tumours.[24,25] *MLK3* oncogenic mutations were found in 21% of the MSI gastrointestinal cases and were described to be functionally relevant.[26]

In the present study, we aimed to: (1) determine the frequency of activating oncogenic gene mutations in the 3′-UTR A13 repeat of *EGFR* in mutation hotspots from *EGFR*, *KRAS*, *BRAF* and *PIK3CA*, as well as in the full coding region of *MLK3*, in a series of 63 MSI GC, and (2) to analyse the pattern of these oncogenic mutations to understand the role played by *EGFR* and its downstream targets, namely those belonging to the *MAPK* and *PI3K* pathways in GC progression. Mutations were screened in all cases and associations between the molecular data and the clinicopathologic features of the patients and tumours were also studied.

2. Patients and methods

2.1. Gastric cancer patients and genomic DNA extraction

To assess MSI frequency, 250 GC patients were analysed.[27–29] In total, we selected a series of 63 MSI GC well characterized in terms of clinicopathologic features and geographic area of origin. Microsatellite analysis was evaluated using five quasi-monomorphic mononucleotide repeats BAT-26, BAT-25, NR-24, NR-21 and NR27 cases were considered MSI whenever two or more markers showed instability on five loci considered.[28] The study population was stratified according to area of residence into Central Italy, representing a GC high-risk area, and Southern Italy, representing a GC low-risk area. Tumour and constitutional DNA were extracted from fresh frozen sample tissues using a standard protocol (Gentra Systems, Minneapolis, USA). Pathological examination allowed the selection of areas of neoplastic cells of more than 80%.

2.2. Somatic mutation analysis of EGFR, KRAS, BRAF, PIK3CA and MLK3 oncogenes

For the *EGFR* gene, direct sequencing of the kinase domain (exons 18, 19, 20 and 21) was performed, using a detailed protocol described by Moutinho and colleagues.[13] Structural alterations on the A13 repeat within the 3′-untranslated region of *EGFR* (3′-UTR polyA repeat) gene were also searched, according with the protocol recently described by Yuan[14] in MSI colon cancer. The 3′-UTR polyA repeat was evaluated in normal, as well as, in GC samples. Mutation analysis of *KRAS* codons 12 and 13 and *BRAF* V600E hotspot mutation were performed by PCR amplification and direct sequencing using the protocol used by Oliveira.[30] To search for somatic alterations of *PIK3CA* gene, exons 9 and 20 were sequenced according to the protocol described in detail by Velho.[18] All exons and intron-exon boundaries of *MLK3* gene were screened for mutations. Primer sequences and PCR conditions adopted were recently described.[26] Except for exon 9, a multiplex PCR approach was used to amplify *MLK3* sequence using the QuantiTect Multiplex PCR Kit (Multiplex PCR, Qiagen, Studio City, CA) and following the manufacturer instructions. Purified PCR products were directly sequenced. All sequence alterations in *EGFR*, *KRAS*, *BRAF*, *PIK3CA and MLK3* genes were validated with a second independent PCR.

2.3. EGFR immunohistochemistry

EGFR immunohistochemistry was evaluated on 3 μm sections from formalin-fixed, paraffin-embedded tissue in only two cases with A13 repeat deletion and in one wild-type sample due to the lack of good quality paraffin material for analysis. Epitope retrieval for *EGFR* was performed by proteolytic enzyme digestion (pepsin A, 4 g/l; Sigma–Aldrich, Germany) at 37 °C. After the antigen retrieval procedure, the slides were washed in a phosphate buffer solution (PBS), and submitted to blockage of the endogenous peroxidase activity by incubation of the slides in a 3% hydrogen peroxide (Panreac, Spain) in methanol (Sigma–Aldrich). The slides were further incubated with a blocking serum (LabVision Corporation kit) for 15 min and then incubated with the primary antibody anti-*EGFR* (Zymed, San Francisco, CA, USA, dilution: 1/100; Clone: 31G7) during 60 min. The secondary antibody was associated with HRP labelled polymer (DakoCytomation) and, after that, the slides were immediately revealed with DAB. Tissues were then counterstaining with Mayer's haematoxylin, dehydrated and coverslipped using a permanent mounting solution

(Zymed, San Francisco, CA, USA). Positive and negative controls were included in order to guarantee the reliability of the assay.

2.4. *Statistical analysis*

Analyses were performed using the Statistical Product and Service Solutions, SPSS 14.0 for Windows, 2006, SPSS Inc., Chicago, IL, USA. Statistical associations between the presence of GC oncogenic mutations and clinicopathologic characteristics was assessed by chi-square test for categorical variables and Student's *t*-test or ANOVA test for continuous variables. A p value lower than 0.05 was considered significant.

3. Results

Thirty-five of 63 (55.6%) MSI GC showed oncogenic mutations in at least one of the screened genes (*EGFR*, *KRAS*, *BRAF*, *PIK3CA* and *MLK3*) (Table 1). Representative images of tumour specific oncogenic mutations are shown in Fig. 1. Table 2, summarizes the association studies performed between the presence of oncogenic mutations (independently of the number and type of genes mutated per case) and the clinicopathologic features of patients and tumours.

3.1. *EGFR screening*

We did not find pathogenic mutations in the hotspot regions of *EGFR* (exons 18, 19, 20 and 21). Heterozygous polymorphic variants of *EGFR* were detected in exon 20 (G>A; rs1050171) and in exon 18 (G>A; rs55959834) in 54 and in 2 samples, respectively. However, deletions at the A13 repeat localized into the 3′ UTR of *EGFR* were found in 30/63 (47.6%) of MSI GC. Within the 30 mutated carcinomas, 15 (50%) showed a mononucleotide A deletion, 12 (40%) had a dinucleotide A deletion and, 3 (10%) a trinucleotide A deletion. All 63 normal

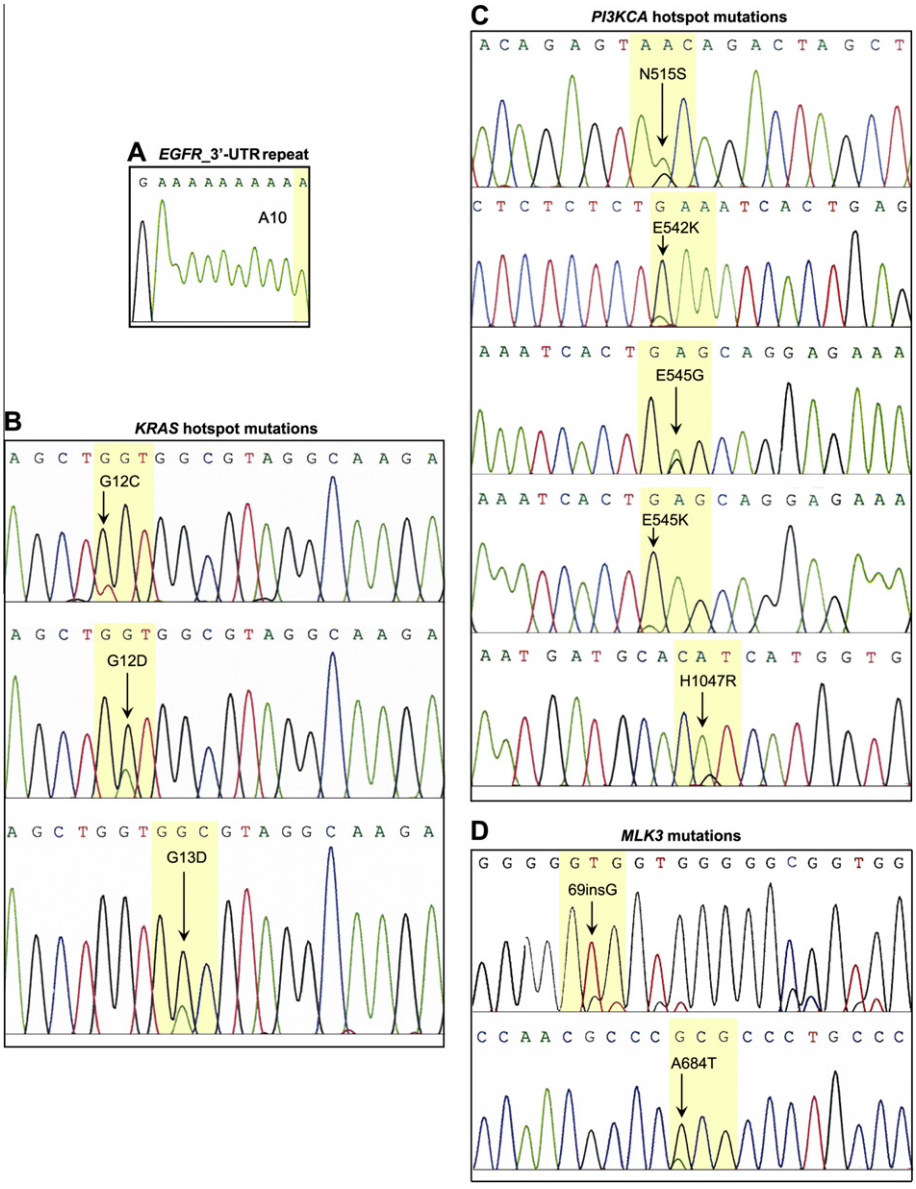

Fig. 1 – Representative images of all oncogenic mutations identified. (A) A10 tumour deletion localized into the 3′ UTR of *EGFR*; (B) *KRAS* hotspot mutations (reverse sequence); (C) *PIK3CA* tumour specific alterations; and (D) *MLK3* gene alterations.

Table 1 – Detailed description of oncogenic mutations identified in the 63 MSI gastric carcinomas.

	Sample code	*MLK3* mutation	*KRAS* mutation	*PIK3CA* mutation	*BRAF* mutation	*EGFR* hotspot mutation	*EGFR* 3′-UTR polyA deletion
1	AU415	wt	wt	**N515S**	wt	wt	$(A)_{13}$ /$(A)_{13}$
2	AB248	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
3	BG191	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{13}$
4	BF070	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{13}$
5	BM175	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
6	BL276	wt	wt	wt	wt	wt	**delA**$(A)_{12}$ /$(A)_{13}$
7	BE163	wt	**G12D**	**H1047R**	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
8	BM213	wt	**G12D**	wt	wt	wt	**delA**$(A)_{13}$/**delA**$(A)_{13}$
9	BC355	wt	**G13D**	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
10	BM406	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
11	BP280	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
12	CD361	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
13	CE120	wt	wt	**H1047R**	wt	wt	**delAAA**$(A)_{10}$/**delAAA**$(A)_{11}$
14	CC442	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
15	CI362	wt	**G12C**	**H1047R**	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
16	CG072	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
17	CI376	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
18	DM272	wt	wt	wt	wt	wt	**delAAA**$(A)_{10}$/**delAAA**$(A)_{10}$
19	DE226	wt	wt	**E545K**	wt	wt	$(A)_{13}$/$(A)_{14}$
20	DM187	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
21	FF336	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
22	FL141	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
23	FL208	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
24	FF269	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
25	FP285	wt	wt	wt	wt	wt	$(A)_{14}$/$(A)_{14}$
26	FD373	wt	wt	wt	wt	wt	$(A)_{1}$ /$(A)_{14}$
27	GA220	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
28	GE126	wt	**G12D**	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
29	GF364	wt	wt	wt	wt	wt	$(A)_{14}$/$(A)_{14}$
30	JG153	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
31	LG445	wt	**G13D**	wt	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
32	LI266	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
33	MA155	wt	**G12D**	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
34	MG296	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
35	MI052	wt	wt	**H1047R**	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
36	MS399	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
37	MG359	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
38	ME196	wt	wt	wt	wt	wt	**delAA**$(A)_{10}$/**delA**$(A)_{12}$
39	MM110	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
40	MM122	wt	**G12D**	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
41	NV424	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
42	NC410	wt	wt	wt	wt	wt	$(A)_{1}$ /$(A)_{13}$
43	OG249	wt	wt	**E545G**	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
44	PA158	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
45	PB114	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
46	PD192	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{11}$
47	PR209	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
48	PD214	wt	**G12D**	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
49	RF421	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{13}$
50	RA125	**A684T**	wt	**E542K**	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
51	RI107	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
52	RA393	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
53	RA270	wt	**G13D**	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
54	RC205	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
55	RL139	wt	wt	**E542K**	wt	wt	$(A)_{13}$/$(A)_{14}$
56	SA13 5	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
57	SE315	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
58	SA259	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
59	TL130	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
60	TB146	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
61	VN174	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
62	VP199	**69insG**	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
63	ZR195	wt	**G13D**	wt	wt	wt	**delAAA**$(A)_{10}$/**delAAA**$(A)_{11}$
Total		2	11	9	0	0	30

Table 2 – Relation between clinicopathologic features of the 63 MSI gastric carcinomas and oncogenic mutations.

	Oncogenic mutation + (35/63; 55.6%)[a]	Oncogenic mutation –(28/63; 44.4%)	P value
Gender			
Male	10 (28.6)	18 (64.3)	0.046
Female	25 (71.4)	10 (35.7)	
Mean age (±SD)	76.7 ± 7.9	67.9 ± 11.4	0.001
Tumor location			
Cardia	4 (11.4)	1 (3.6)	ns
Non-cardia	31 (88.6)	27 (96.4)	
Lauren classification			
Intestinal	31 (88.6)	19 (67.9)	0.043
Non-intestinal	4 (11.4)	9 (32.1)	
Depth of invasion			
pT1–T2	25 (71.4)	14 (50)	ns
pT3–pT4	10 (28.6)	14 (50)	
Lymph node involvement			
pN0–N1	30 (85.7)	20 (71.4)	ns
pN2–pN3	5 (14.3)	8 (28.6)	
Extent of gastrectomy			
Partial	27 (77.1)	20 (71.4)	ns
Total	8 (22.9)	8 (28.6)	
Lymphadenectomy			
D1	15 (42.8)	12 (42.9)	ns
D2/D3	20 (57.2)	16 (57.1)	
Radicality of resection			
R0	32 (91.4)	19 (67.9)	0.017
R1–2	3 (8.6)	9 (32.1)	
Stage grouping			
I–II	23 (65.7)	14 (50)	ns
III–IV	12 (34.3)	14 (50)	
Geographic area			
High incidence	35 (100)	21 (75)	0.001
Low incidence	0 (0)	7 (25)	

Numbers in parentheses are percentage.
[a] Fourteen GC patients carried concomitant somatic mutations.

gastric samples showed a wild-type A13 or A14 repeat of *EGFR* in a homozygous or heterozygous state. According with the findings from the *EGFR* 3′-UTR mutation screening, we verified, by *EGFR* immunohistochemistry, that while two *EGFR* mutant GC (deletion [delAA(A)11]) showed an increased expression of *EGFR* in tumour cells in comparison to the surrounding normal tissue, a wild-type *EGFR* GC case did not display *EGFR* expression in the tumour area (Fig. 2). Due to the lack of good quality paraffin tissue, we can only consider this data as preliminary. This result needs to be validated in a larger series of MSI GC in order to confirm the correlation between the *EGFR* expression and the mutations in A13 deletions in the 3′-UTR polyA repeat of EGFR.

3.2. *KRAS and BRAF mutations*

KRAS mutations were observed in 17.5% (11/63) of MSI GC cases. From these 11 cases harbouring somatic mutations, we found that codon 12 was mutated in 63.6% (7/11) and codon 13 in 36.4% (4/11). All but one *KRAS* mutation localized in codon 12 were G12D. All mutations in codon 13 were G13D. *KRAS* mutations were more frequently found in elderly patients ($p = 0.006$), but no further significant associations were found between other clinicopathologic characteristics and the *KRAS* mutation status (data not show). None of the MSI GC cases under study showed *BRAF* somatic mutations in the hotspot codon previously associated to MSI colorectal carcinomas, the *BRAF* V600E.[31]

3.3. *PIK3CA mutations*

PIK3CA mutations were found in 14.3% (9/63) of the MSI GC studied. Among the mutated cases, eight somatic mutations were located in codons previously described as *PIK3CA* hotspots (codons 542, 545 and 1047). Five *PIK3CA* alterations occurred at the helical domain (codon 515, 542 and 545) and four mutations affected the kinase domain (codon 1047) (Table 1). A novel missense mutation (1544A>G) was identified within the helical domain at codon 515 (N515S). None of these mutations were present in the normal counterpart of these cases.

3.4. *MLK3 mutations*

MLK3 mutations were found in two carcinomas corresponding to a frequency of 3.2% (Table 1): one was a missense mutation (2052G>A) localized in proline-serine-threonine rich domain (A684T) and the other was a frameshift mutation (c.69insG). Besides these two somatic mutations, that were tumour specific, we identified one splice site alteration (c.1069+10C>T) and one missense mutation (2190G>A) (R730H) that were present in both tumour and constitutional DNA, both with unknown pathogenic function. Moreover, we observed two silent *MLK3* sequence variants (225A>G and 2259A>T) that did not change the aminoacid residues of the *MLK3* protein (A75A and P753P, respectively) and were previously described in the normal population.[26]

3.5. *Concomitant oncogenic mutations*

We verified that within the 35 MSI GC harbouring oncogenic mutations, 14 (40.0%) of the mutant cases showed concomitant oncogenic alterations, always involving *EGFR* polyA mutations. From those cases with more than one mutation, seven had *EGFR* A13 repeat deletions and *KRAS* mutations (7/14 – 50%), three showed *EGFR* polyA deletion and a *PIK3CA* mutation (3/14 – 21.4%) and two had *EGFR* deletions and both *KRAS* and *PIK3CA* (2/14 – 14.3%) mutations. In one case we found an *EGFR* deletion and concomitant missense mutations in *MLK3* and *PIK3CA* and in another case an *EGFR* deletion and a *MLK3* mutation (Fig. 3).

4. Discussion

Despite the general advances in diagnosis, standard surgery and chemo- and radio-therapy regimens, the overall outcome of GC patients remains poor, with a 5-year global survival of

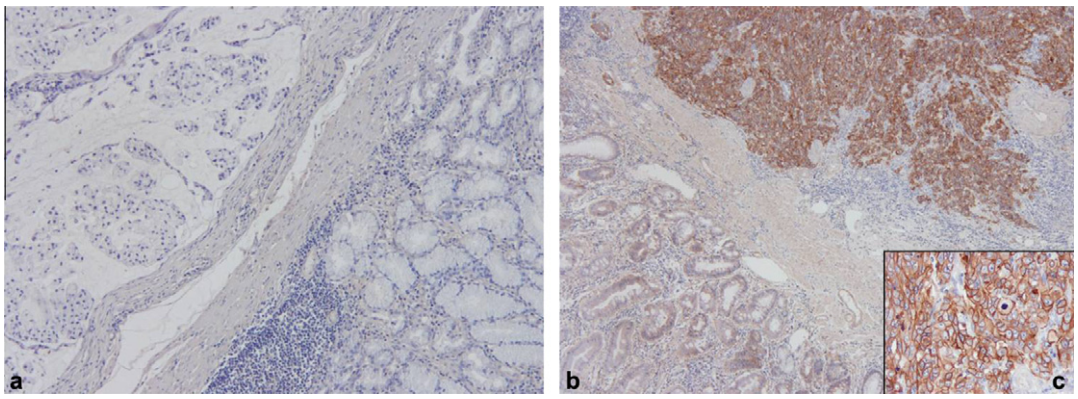

Fig. 2 – *EGFR* immunohistochemistry expression in gastric carcinoma: (a) example of a *EGFR* A13 tract wild-type GC case showing negative *EGFR* expression (amplification 100X); (b) *EGFR* overexpression in tumour area in a GC case displaying *EGFR* A10 deletion in 50× amplification and (c) in 400× amplification.

about 26%.[32] Various multimodal therapy regimens are used to improve the prognosis of GC patients, but no single chemo-therapy regimen is recognized as a global standard.[33] The high prevalence of incurable disease and the poor overall survival of GC patients create the urgent need to find new therapeutic tools for GC treatment.

In patients with advanced GC, several clinical trials were conducted, namely testing *EGFR* inhibitors. However, the overall response to *EGFR* tyrosine kinase inhibitors was poor. Among *EGFR* tyrosine kinase inhibitors, it has been demonstrated that gefinitib treatment showed a particularly low response rate (18%) in advanced GC while erlotinib was completely inactive.[34,35] Recently, more encouraging results were obtained in GC treatment using a combination of multiple chemotherapies.[36–41] As example, patients treated with cetuximab and FUFOX/FOLFOX (5-fluorouracil, oxaliplatin, folinic acid) or FOLFIRI (5-fluorouracil, irinotecan and folinic acid) had a significant higher response rate (62%) when compared with GC patients treated with cetuximab alone (5%).[36–38] Furthermore, when cetuximab was associated with oxaliplatin/leucovorin and 5-fluorouracil, 50% of the patients showed a positive response rate.[39,40] A similar response rate (65%) has been reported in metastatic GC treated with cetuximab plus oxaliplatin/ folinic acid, in which *EGFR* overexpression has been documented.[41] However, until now it was impossible to predict which GC patients will respond to anti-*EGFR* therapy. In other words, no predictive biomarkers are available for clinicians to use in the stratification of GC patients or to predict treatment benefit. Taking into account data from treatment of colon cancer patients, where the response to anti-*EGFR* therapy depends on the genetic make-up of the tumours, namely on the *EGFR* activation status and on the mutation profile of members of *EGFR* and *MAPK* signalling pathways,[42,43] we decided to study these same players in MSI GC.

Different molecular mechanisms underlie *EGFR* protein activation, such as somatic mutations or gene amplification, both leading to an abnormal receptor function. In our series

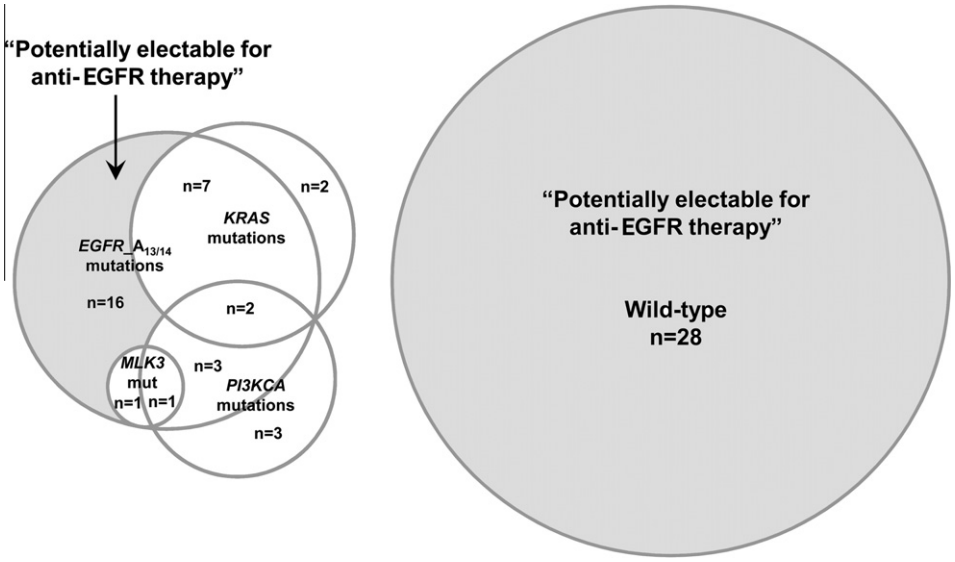

Fig. 3 – Schematic representation of the distribution of the oncogenic mutations identified in the present study (occurring as single events or as concomitant alterations). Oncogenic mutations were found in 35 GC cases but fourteen of them showed more than one mutagenic event in genes belonging to the *EGFR* signalling pathway.

of MSI GC, we did not find hotspot *EGFR* mutations. This finding was not surprising as *EGFR* mutations have been rarely described in GC and gene amplification was only described in a low frequency of cases.[13] Therefore, the presence of other mechanisms directly or indirectly leading to *EGFR* activation were herein investigated.

Since it was recently demonstrated that a polyA tract at the 3′-UTR of *EGFR* was prone to harbour deletions in MSI colon cancer,[14] we searched for this type of alterations in our series of MSI GC. We found alterations at this site in a high frequency (47.6%) of cases. A higher frequency (69%) was found for this type of alteration in MSI colon cancer.[14] Furthermore, and similarly to *EGFR* 3′-UTR polyA tract mutant colon cancer cases, we also verified high level of *EGFR* expression in two tumour samples harbouring this type of alteration. These results suggest *EGFR* A13 repeat mutations as a putative molecular marker to select GC patients for anti-*EGFR* therapies, namely anti-*EGFR* monoclonal antibodies as verified in colon cancer.[14,45]

As previous mentioned, in metastatic colorectal carcinomas, a response to anti-*EGFR* therapies was only observed in patients with tumours without *KRAS* or *BRAF* mutations.[4,6,17,43–45] In particular, the presence of a *KRAS* mutation is well established as predictive of non-response to anti-*EGFR* antibody and shorter patient survival.[5,6] In contrast, the clinical evidence concerning *PIK3CA* mutations is not yet straightforward in this cancer setting.[46,47]

Given the similarities in the mutation spectrum of colorectal carcinomas and MSI gastric carcinomas,[18] we hypothesize that MSI GC cases without alterations in *KRAS* and *BRAF*, and possibly in other members of the *MAPK* (*MLK3*) or *PI3K* (*PIK3CA*) pathways may benefit from this therapeutic approach. Taking in account this hypothesis, we determined the frequency of mutations in all these genes in order to verify their putative importance as biomarkers for therapy assessment.

In the present study, *KRAS* mutations were found in 17.5% of MSI GC cases, while no *BRAF* mutations were detected in these samples, in keeping with data on record.[15,16,18,20–22] These data elicit *KRAS* but not *BRAF* activation as potential biomarkers for therapy assessment in MSI GC. Similarly to *BRAF*, somatic alterations at the *MLK3* gene were very rarely found (3.2%), as previously described in another sample setting of MSI GC.[26] *PIK3CA* mutations were found in a non-neglectable frequency of MSI GC cases (14.3%, respectively) and despite no proof exists showing that mutant *PIK3CA* tumours may not respond to anti-*EGFR* therapy, it is important to characterize its mutation status. The above presented data suggest, in light of the current knowledge on the treatment of gastrointestinal cancer with anti-*EGFR* therapy, that MSI GC patients: (1) can also be stratified according to the molecular profile of their tumours; (2) present, in over 45% of the cases mutations at the *EGFR* 3′-UTR that potentially lead to *EGFR* overexpression which turn these cases in a subset of potentially responsive tumours to anti-*EGFR* treatment; (3) display *KRAS* mutations in nearly 20% of the cases making these GC patients as potentially resistant to anti-*EGFR* therapies; (4) harbour *PI3KCA* mutations in almost 15% of the cases but the use of this information is of limited interest, and finally; (5) lack or display very low frequencies of *BRAF* and *MLK3* mutations and therefore these markers will not improve a future panel of genes to be tested in potentially eligible patients for anti-*EGFR* therapy. Another layer of information that was obtained in our multiple gene mutation screening approach, showed that over one third (40%) of the mutant MSI GC cases accumulate mutations in more than one gene, demonstrating, on one hand, that multiple molecules within or targeted by the *EGFR* pathway are involved in GC progression; but on the other hand, reducing the number of cases potentially benefitting from anti-*EGFR* therapy. In detail, as 30% (9/30) of *EGFR* mutated cases also display *KRAS* mutations, thus it is predictable that 70% (21/30) of mutant *EGFR* patients will benefit from *EGFR* inhibitors (Fig. 3). Furthermore, another set of the patients harbouring *EGFR* mutations also displayed a *PIK3CA* mutation which, although unproved, may also result in resistance to therapy (Fig. 3).

In GC, cases harbouring concomitant oncogenic mutations in the *MAPK* cascade is about 3%.[18] Our data showed that 40% of cases have concomitant oncogenic mutations (Fig. 3). In our series, the higher percentage of cases with multiple oncogenic mutations is related to the additional screening of the *EGFR* polyA tract and *MLK3* gene, never evaluated so far. In fact, considering only *PIK3CA* and *KRAS* alterations, the number of cases with concomitant mutations is much lower and similar to the frequency reported by Velho and colleagues.[18] The accumulation of *EGFR* 3′-UTR and/or *PIK3CA*/*KRAS*/*MLK3* mutations within MSI gastric carcinomas suggest a possible synergistic effect in the signalling pathways associated to the activation of these genes in GC development/ progression.

Our results support the proposal to implement a multigene screening approach to predict *EGFR*-targeted therapy. We show that within MSI GC three groups can be individualized: Group A: cases that are wild-type for all genes under screening (*EGFR* and genes of the *MAPK* and *PI3K* pathways) – 44.4% (28/63); Group B: cases with oncogenic alterations restricted to *EGFR* - 25.4% (16/63); Group C: cases with oncogenic mutations in genes of the *MAPK* and *PI3K* pathways with or without concomitant *EGFR* alterations -30.2% (19/63). Overall, cases from groups A and B (69.8%) are potentially eligible for anti-*EGFR* therapy. Moreover, from the experience with colorectal cancer it is expectable that cases with oncogenic mutations affecting *MAPK* and *PI3K* signalling pathways GC patients will be non-responders for anti-*EGFR* therapies.

The advantage to identify deletion at the *EGFR* A13 repeat region is to select a novel category of patients that potentially benefit from *EGFR* inhibitors as therapeutic approach. Probably other genetic mechanisms, to date unknown, can also activated the *EGFR* pathway even in cases that belong, in our series, to the wild-type *EGFR* group.

We verified that oncogenic mutations mostly occur in MSI GC patient with older age at diagnosis and of the intestinal subtype, supporting that within MSI GC different molecular pathways are activated in order to generate specific phenotypes.

Palli and colleagues showed in another study performed in the same geographical region (Tuscany region) an association between MSI, positive family history of GC and high consumption of red meat and nitrates, suggesting that environmental factors, such as nutritional habits may play a key

role in inducing genomic instability and an increased risk for GC. Our data demonstrated that oncogenic mutations related significantly with this high incidence area and this probably is associated with the presence of MSI phenotype.[27,48]

In conclusion, our results show that alterations at multiple molecules within or targeted by the *EGFR* pathway are frequent in MSI GC and, that within members of the pathway, deletions of the A13 repeat of *EGFR* were the most common genetic event followed by *KRAS* and *PIK3CA* mutations. Furthermore, in over one third of the cases, concomitant mutations occur in distinct genes, always involving *EGFR*. More importantly, our results open new avenues regarding the stratification of MSI GC patients for anti-*EGFR* therapies and pinpoint a non-neglectable group of cases that may benefit from this therapeutic approach.

Conflict of interest statement

None declared.

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