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Oncogenic mutations in gastric cancer with microsatellite instability

Giovanni Corso [a,b,c], *Sérgia Velho* [a], *Joana Paredes* [a,d], *Corrado Pedrazzani* [b,c], *Diana Martins* [a], *Fernanda Milanezi* [a], *Valeria Pascale* [b], *Carla Vindigni* [e], *Hugo Pinheiro* [a], *Marina Leite* [a], *Daniele Marrelli* [b,c], *Sónia Sousa* [a], *Fátima Carneiro* [a,d,f], *Carla Oliveira* [a,d], *Franco Roviello* [b,c], *Raquel Seruca* [a,d,*]

[a] *Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Portugal*
[b] *Department of Human Pathology and Oncology, Section of General Surgery and Surgical Oncology, Translational Research Laboratory, University of Siena, Italy*
[c] *Istituto Toscano Tumori (ITT), Firenze, Italy*
[d] *Faculty of Medicine of the University of Porto, Portugal*
[e] *Unit of Pathology, Azienda Ospedaliera Universitaria Senese, Siena, Italy*
[f] *Department of Pathology, Hospital S. João, Porto, Portugal*

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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.

* *Corresponding author:* Address: Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal. Tel.: +351 225570700; fax: +351 225570799.
E-mail address: rseruca@ipatimup.pt (R. Seruca).

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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