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Original contribution

Analysis of *KRAS*, *NRAS*, *PIK3CA*, and *BRAF* mutational profile in poorly differentiated clusters of KRAS-mutated colon cancer☆,☆☆

Luca Reggiani Bonetti MD, PhD[a,*,1], Valeria Barresi MD, PhD[b,,1], Stefania Bettelli BSc[a], Cecilia Caprera BSc[a], Samantha Manfredini BSc[a], Antonio Maiorana MD[a]**

[a]*Department of Diagnostic Medicine and Public Health, , University of Modena and Reggio Emilia—Section of Pathology, University of Modena and Reggio Emilia, 41100 Modena, Italy*
[b]*Department of Human Pathology, University of Messina, 98125 Messina, Italy*

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Summary Recently, a grading system based on the counting of poorly differentiated clusters (PDCs) of neoplastic cells was shown to be a strong predictor of nodal metastases and negative prognosis in colon cancer (CC). In this study, we assessed and compared the mutational status of *KRAS*, *NRAS*, and *PIK3CA* in PDCs and corresponding main tumor tissue of 25 CCs with *KRAS* mutations. For each tumor, PDC and main tumor tissue were distinctly analyzed by using laser microdissection and mass spectrometry. In 3 CCs, the main tumor tissue had also *PIK3CA* mutations (C420R: 1; E545K: 1; H1047R: 1), and in 1, it showed *NRAS* mutation (codon 12). In 20 cases, PDCs had the same biomolecular profile as the main tumor, but in 5, they had different biomolecular profiles. In detail, PDCs had *KRAS* wild type in 2 cases and additional *PIK3CA* mutations (E542K: 1; H1047Y: 1; E545Q: 1) in 3. All 3 cases with additional *PIK3CA* mutations in PDCs had nodal metastases, high pathological TNM stage, and lymphatic invasion. In 1 of 3 cases, additional *PIK3CA* mutation detected in PDC, but not in the main tumor, was also found in the corresponding nodal metastases. Our findings show for the first time that heterogeneous biomolecular profile previously observed in CC may depend on different histologic aspects of the lesion. Because PDCs may represent the tumor cells with the highest potential to metastatize, their molecular status may be relevant for the prediction of response to targeted therapies.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and a leading cause of cancer-related death worldwide [1]. At present, the pathological TNM (pTNM) stage assessed in accordance to the International Union Against Cancer TNM [2] and to the American Joint Committee on Cancer [3] represents the most relevant prognostic factor for CRC [4] and determines its postsurgical treatment. However, in some cases,

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* Correspondence to: L. Reggiani Bonetti, Department of Diagnostic Medicine and Public Health, University of Modena and Reggio Emilia Section of Pathology, Via del Pozzo 71, 41100 Modena, Italy.
** Correspondence to: V. Barresi, Department of Human Pathology, Policlinico G. Martino, Via Consolare Valeria, 98125 Messina, Italy.
E-mail addresses: reggiani.luca@policlinico.mo.it (L. Reggiani Bonetti), vbarresi@unime.it (V. Barresi).
[1] These authors contributed equally to this work.

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pTNM stage has no correlation with the biological and clinical behavior of CRC [5,6]. Hence, the identification of additional factors, which may predict the clinical course of CRC with more accuracy and regardless of pTNM stage, has been a main research focus over the years.

Recently, ours and other groups showed that a novel grading system based on the number of clusters composed of at least 5 cancer cells and lacking a gland-like structure—so-called poorly differentiated clusters (PDCs)—represents a significant prognostic factor in patients with CRC, independently of pTNM stage [6-13]. In addition, the presence of a high number of PDCs was significantly associated with higher metastatic risk of CRC [7-12]. This association may be explained by the relationship between PDC and epithelial-mesenchymal transition (EMT) [14,15], a process by which neoplastic cells lose epithelial properties and acquire the mesenchymal cell potential to migrate through the extracellular matrix and to metastatize. Indeed, PDCs show several characteristics of EMT phenotype, such as reversed pattern of MUC1 expression, loss of or aberrant E-cadherin expression, up-regulation of *WNT/β*-catenin signaling pathway, and matrix metalloproteinase expression [15-17]. Thus, they have invasive properties similar to those seen in cells forming tumor budding foci [15]. However, because they are composed of at least 5 neoplastic cells [7], PDCs are more easily identified with conventional hematoxylin and eosin stain [18].

In a recent study, we found that CRCs with high counts of PDCs display mutations in *KRAS* gene with significantly higher frequency than CRCs with absent/low number of PDCs [19]. We speculated that this association may depend on the ability of *RAS* oncogenic mutations to induce dedifferentiation of CRC cells and EMT process [20,21]. However, whether *KRAS* mutations actually exert a role in driving PDC formation is still to be determined. In addition, the molecular signature of PDCs has not been analyzed thus far.

On this premise, aims of the present study were as follows: (*a*) to investigate the mutational status of genes involved in the *RAS/MAPK* and *PI3K-PTEN-AKT* signaling pathways in the PDCs of a series of colonic carcinomas (CCs) showing *KRAS* mutations and (*b*) to compare the mutational status observed in PDCs with that revealed in the corresponding main tumor.

2. Materials and methods

Twenty-five consecutive *KRAS*-mutated surgically resected CCs with more than 10 PDCs at the invasive front of growth (PDC G3 tumors) were taken from the files of the Unit of Pathological Anatomy of Modena, Italy. All cases were anonymously collected, and all procedures were performed in accordance with the Helsinki Declaration. All relevant issues were discussed with the local ethics committee that established that no further ethical approval was needed to revise histology or to perform molecular analyses in the cases included in the study.

In all cases, PDC grading had been assessed as previously described [6-12]. First, the whole tumor had been scanned at lower-power magnification to identify the area with the highest number of PDCs. Then, the clusters had been counted under the microscopic field of a ×20 objective lens (ie, a microscopic field with a major axis of 1 mm), using a Zeiss microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany). The maximum number of PDCs found in all sections had been considered for grading. More specifically, cancers with less than 5, 5 to 9, and at least 10 clusters had been classified as grade 1 (G1), grade 2 (G2), and grade 3 (G3), respectively [7]. For each case, data on the localization in the large bowel, World Health Organization (WHO) histologic grade [4], pTNM stage [2,3], and presence of tumor budding and lymphovascular invasion (LVI) were available.

2.1. Molecular analysis

In all cases, mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes were separately analyzed in the main tumor and in their PDC as well. Mutational status of the same genes was also investigated in the nodal metastases of CCs showing discordance in *PIK3CA* status between PDC and main tumor. Slides 10 μm thick were cut from the representative paraffin blocks of each primary tumor or nodal metastases. For each case, all PDCs (at least 10) were microdissected using Laser Microdissector CKX41 (Olympus, Tokyo, Japan) and collected in specific tubes (Fig. 1). In all the cases, PDCs were present only in the invasive front of the tumor and dissected from there. Then, tumor cells of the main tumor mass were collected in different tubes. All samples were deparaffinized, and DNA extraction was performed in lysis buffer (50 mM Tris, 1 mM EDTA, 0.5% Tween 20) with the addition of proteinase K (10 mg/mL) overnight at 56°C. Proteinase K was inactivated at 95°C twice for 10 minutes. DNA was quantified with Xpose-NGS (Trinean NV, Gentbrugge, Belgium). Oncogenic mutations of *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes were analyzed with Myriapod Colon Status Kit (Diatech Pharmacogenetics, Jesi, Ancona, Italy) using the high-throughput genotyping platform Sequenom MassARRAY System (Sequenom, San Diego, CA). The molecular array allows for identification of the most important mutations of *KRAS* (codons 12, 13, 59, 61, 117, 146), *NRAS* (codons 12, 13, 18, 59, 61, 117, 146), *BRAF* (codons 594, 600, 601), and *PIK3CA* (codons 38, 81, 88, 93, 108, 118, 345, 420, 539, 542, 545, 546, 549, 1021, 1025, 1043, 1047, 1049). In brief, 25 ng/μm DNA of each primary tumor and corresponding PDC was amplified with multiplex polymerase chain reaction and then treated with shrimp alkaline phosphatase to neutralize unincorporated deoxyribonucleotide triphosphate; afterward, the mutation site was extended with modified primers and the samples were read with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. Analysis was performed using Typer 4.0 software (Sequenom, San Diego, CA), which allows for the mutated allele identification by comparing ratios of the wild-type (WT) peak of all suspected mutants and generates a specific report.