Tumor-infiltrating lymphocytes and dendritic cells in human colorectal cancer: Their relationship to KRAS mutational status and disease recurrence

Petr Kocián a,b, Monika Šedivcová c, Jan Drgáč d, Kateřina Černá e, Jiří Hoch b, Roman Kodet d, Jiřina Bartůnková a, Radek Špišek a, Anna Fialová a,*

* Department of Immunochemistry, 2nd Faculty of Medicine, Charles University and University Hospital Motol, V Úvalu 84, 15006 Prague 5, Czech Republic
b Department of Surgery, 2nd Faculty of Medicine, Charles University and University Hospital Motol, V Úvalu 84, 15006 Prague 5, Czech Republic
c Department of Pathology, Medical School and University Hospital, Charles University, 304 60 Pilsen, Czech Republic
d Department of Pathology and Molecular Medicine, 2nd Faculty of Medicine, Charles University and University Hospital Motol, V Úvalu 84, 15006 Prague 5, Czech Republic

Article history:
Received 19 March 2011
Accepted 25 July 2011
Available online 16 August 2011

Keywords:
Colorectal cancer
KRAS
Tumor-infiltrating lymphocytes

ABSTRACT

The prognosis of newly diagnosed colorectal cancer patients relies mostly on tumor-node metastasis classification. However, analyses of tumor-infiltrating lymphocytes and several molecular markers have also shown promising prognostic value. Mutations in the proto-oncogene KRAS, which occur early in colorectal carcinogenesis, have been demonstrated to be common in human colorectal cancer (CRC); however, their prognostic significance remains controversial. We examined the correlations between KRAS mutational status and tumor-infiltrating immune cells with respect to CRC recurrence. Mutations in KRAS were identified in 45.5% of the primary carcinomas in our cohort of patients: 65% in codon 12 and 35% in codon 13. Although codon 13 KRAS mutations were associated with disease relapse, they were present in both disease-free and relapsed patients. However, disease-free and relapsed patients differed markedly in their patterns of tumor-infiltrating immune cells. There was a trend toward decreased density of tumor-infiltrating lymphocytes (TILs) within the group of relapsed cases. In addition, relapsed patients with codon 13 mutations had markedly lower levels of tumor-infiltrating mature DC-LAMP+ dendritic cells (DCs) and higher frequency of CD1a+ cells compared with disease-free patients. Our data suggest that CRC patients with low levels of TILs, a high CD1a+/DC-LAMP+ tumor-infiltrating DC ratio, and a KRAS mutation in codon 13 are at a high risk of disease recurrence.

© 2011 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.

1. Introduction

Colorectal cancer (CRC) is one of the 3 most common malignant neoplasms worldwide, with an incidence of 1.2 million cases per year. The estimates for CRC-related deaths are more than 600,000 annually [1]. Despite the recent progress in diagnosis and treatment, the prognosis of advanced CRC remains poor, often with palliative therapy as the only option. Because the prognostic value of the standard tumor-node metastasis (TNM) staging system has been recently challenged in many ways, superior prognostic markers are needed to more precisely define prognosis and better predict the benefits of adjuvant treatment in colorectal cancer [2].

Cancer development is a multistep process that involves chromosomal abnormalities and mutations, as well as epigenetic modifications of genes that regulate cell proliferation, differentiation, and apoptosis [3,4]. One of the best known CRC-associated proto-oncogenes is KRAS, which encodes a small, 21-kDa GTP/GDP-binding protein involved in the regulation of the cellular response to a wide range of extracellular stimuli [4,5]. Specific KRAS mutations lead to the constitutive activation of multiple signaling pathways, including 1 downstream of the epidermal growth factor receptor (EGFR), thus driving the growth and progression of CRC and providing an escape mechanism that allows the tumor cells to overcome the pharmacologic inhibition induced by anti-EGFR molecules [4,6]. Activating mutations in KRAS have been observed in 30 to 50% of CRCs [7–9], and up to 90% of these mutations have been reported in codons 12 and 13 [10]. Although a wide range of studies and meta-analyses concerning the prognostic significance of KRAS mutations have been published, the results remain controversial, with only some of them indicating an impact of mutant KRAS on clinical outcome [4,11].

In addition to genetic mutations and TNM staging, a quantitative assessment of the immune cells that infiltrate the tumor tissue and peritumoral areas has been proposed as an independent outcome predictor. Indeed, it has been convincingly demonstrated that high densities of tumor-infiltrating lymphocytes (TILs) are associated with improved recurrence-free and/or overall survival [12–16]. Immune surveillance is believed to play a crucial role in cancer development and progression. The correlation between the pres-
ence of TILs in tumor tissue and improvements in survival supports this concept. Nevertheless, multivariate analyses concerning the densities of tumor-infiltrating immune cells and clinicopathologic data have mostly not included any differences in the molecular phenotype of the patients. In fact, the association between the densities of tumor-infiltrating immune cells and KRAS mutation status has not yet been evaluated. To extend the knowledge about suggested prognostic factors, we examined the correlations between the KRAS mutational status, patterns of tumor-infiltrating immune cells, and the presence of tumor recurrence in a cohort of newly diagnosed CRC patients.

2. Subjects and methods

2.1. Patients and tissue samples

Formalin-fixed paraffin-embedded specimens were obtained from 44 patients with surgically resected colorectal cancer treated between 2004 and 2009 at the University Hospital Motol in Prague, Czech Republic. None of the patients had received neoadjuvant radiotherapy or chemotherapy. The histopathologic stages of the tumors were determined according to the TNM classification system of the Union for International Cancer Control and the American Joint Committee on Cancer. The pathology of each tumor sample was reevaluated by an experienced pathologist. No patient was lost to follow-up. The median duration of follow-up was 55 months for disease-free patients and 20 months for relapsed patients. The collection of all tissue specimens was approved by the Institutional Review Board of the University Hospital Motol. The clinicopathologic characteristics of the patients are summarized in Table 1.

2.2. Detection of KRAS gene mutations

Formalin-fixed paraffin-embedded tumor tissue sections were deparaffinized and genomic DNA was extracted using a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. To detect the mutational status of the KRAS gene, polymerase chain reaction (PCR) amplification of exon 1 was performed using the following primers: sense, 5’-tcattatttttataaggctgccttg-3’; and antisense, 5’-agatgtggtcgcaggactagta-3’. Amplified PCR products were purified by Montage PCR filter units (Millipore, Billerica, MA) and sequenced using a Big Dye Terminator sequencing kit (PE/Applied Biosystems, Foster City, CA) on an automated sequencer ABI Prism 3130xl (PE/Applied Biosystems). The sensitivity of this method is only 10% of mutated cells; therefore, negative samples were subsequently retested using the highly sensitive (1% of mutated alleles) PGX-KRAS-BRAF StripAssay (ViennaLab, Vienna, Austria) according to the manufacturer’s instructions. Briefly, biotinylated multiplex PCR amplification products were hybridized to nitrocellulose test strips and bound sequences were visualized using a streptavidin–alkaline phosphatase conjugate and color substrate.

2.3. Immunofluorescence

For immunostaining, 4-μm-thick sections were cut, deparaffinized using xylene, and subjected to heat antigen retrieval using Tris/EDTA buffer, pH 9 (Dako, Glostrup, Denmark), in a water bath for 35 minutes at 98°C. To reduce the nonspecific background signal, the sections were subsequently treated with Image-IT FX signal enhancer (Invtrogen, Carlsbad, CA) and stained with primary monoclonal antibodies against CD3 (F7.2.38, Dako), CD8 (C8/144B, Dako), FoxP3 (236 A/E7, Abcam), CD1a (CD1a007, Abcam), DC-LAMP (1010E1.01, Dendritics, Lyon, France), cytokeratin (MNF116, Dako, or rabbit polyclonal, Abcam), and Ki-67 (SP6, Abcam) overnight at 4°C. Incubation with Alexa Fluor 488-labeled goat antimouse, Alexa Fluor 488-labeled goat antirabbit (both from Invitrogen), and DyLight 594-labeled goat antirabbit (Jackson ImmunoResearch, Suffolk, UK) secondary antibodies was performed for 45 minutes at room temperature. Stained slides were mounted using ProLong Gold antifade reagent with 4’6-diamidino-2-phenylindole (Invitrogen).

2.4. Quantification of tumor-infiltrating immune cells

Each tumor section was evaluated for lymphocyte or dendritic cell (DC) infiltration in the tumor epithelium and tumor stroma in 10 representative visual fields selected for the most abundant immune cell distribution under a fluorescent microscope (Olympus FV300; Olympus, Hamburg, Germany) at 400× magnification. The proportion of Ki67+ tumor cell nuclei was evaluated in 5 representative fields at 600× magnification, and only epithelial cells were included in the analysis. The count was performed by a single investigator (PK) without knowledge of the clinical outcome or the KRAS mutational status of the patients. Images were obtained using an Olympus FV300 microscope and a DP50 digital camera (Olympus). Representative images for each marker used are shown in Fig. 1.

2.5. Statistical analysis

Statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK). Correlations between tumor-infiltrating immune cells were evaluated using a correlation matrix. Differences between KRAS mutants and wild-type patients with respect to tumor recurrence were estimated using Pearson’s χ² test. Differences between relapsed and disease-free patients were analyzed using the Mann–Whitney U test. The remaining data were analyzed using analysis of variance (ANOVA) followed by Tukey’s HSD test. Additionally, multivariate analysis with Cox regression was performed to assess the prognostic value of tumor-infiltrating immune cells, KRAS mutational status, and stage of the disease for disease-free survival. The results were considered a trend when p < 0.1 and statistically significant when p < 0.05.
3. Results

3.1. Intratumoral distribution of TILs and DCs

Tumor-infiltrating lymphocytes stained with CD3, CD8, or FoxP3 were identified in all of the primary colorectal tumor samples analyzed in this study. Similarly, DC-LAMP-expressing mature DCs were present in 43 (97.7%) tumor samples, whereas CD1a-positive DCs were identified in 42 (95.5%) samples. Representative images of immune cell infiltration are presented in Fig. 1. Markedly higher infiltrations of immune cells were observed in the tumor stroma than in the tumor epithelium (Fig. 2).

The number of FoxP3-expressing cells in the epithelium was positively correlated with the proportion of Ki-67 tumor cell nuclei (correlation coefficient 0.35; \( p = 0.04 \)) and the number of CD8+positive DCs were identified in 42 (95.5%) samples. Representative images of immune cell infiltration are presented in Fig. 1. Markedly higher infiltrations of immune cells were observed in the tumor stroma than in the tumor epithelium (Fig. 2).
cells was negatively correlated with the number of CD1a⁺ DCs in the tumor stroma (correlation coefficient $-0.34; p = 0.046$). We did not observe any significant association between the number of tumor-infiltrating immune cells and the tumor stage; however, our data suggest that the CD1a⁺/DC-LAMP⁺ cell ratio increases during disease progression (Fig. 2).

3.2. Tumor-infiltrating immune cells and tumor cell proliferation status with respect to KRAS mutations

In our cohort, KRAS mutations were identified in 45.5% ($n = 20$) of patients: 65% of mutations were reported in codon 12 and 35% were observed in codon 13. The most frequent mutation in codon 12 resulted in the replacement of a glycine with valine (46.1%). The other mutations resulted in the replacement of a glycine with aspartate (30.8%) or cysteine (23.1%). The most frequent mutation in codon 13 resulted in the replacement of a glycine with aspartate (85.7%); 1 patient (14.3%) had a glycine replaced with cysteine. We observed no significant association between the number of tumor-infiltrating immune cells and the KRAS mutational status; however, we observed that patients with mutations in codon 13 of the KRAS gene had a significantly higher proportion of Ki-67⁺ tumor cells than did wild-type (WT) patients and patients with mutations in codon 12 (Fig. 3A). Moreover, we demonstrated that 57% of patients with mutations in codon 13 experienced disease relapse compared with only 33.3 and 30.1% of WT patients and patients with mutations in codon 12, respectively (Fig. 3B).

3.3. Differences in the characteristics of the immune cell infiltrate between patients with mutations in codon 13 of the KRAS gene with and without disease recurrence

Despite being more abundant in patients with disease recurrence, mutations in codon 13 of the KRAS gene were present in both disease-free and relapsed patients in our cohort. Therefore, we evaluated the characteristics of tumor-infiltrating immune cells in patients with a KRAS mutation in codon 13 with respect to tumor recurrence. We identified a trend toward disease recurrence in patients with low numbers of TILs in both the tumor epithelium and the stroma. Moreover, patients with tumor recurrence had more CD1a⁺ DCs and significantly fewer DC-LAMP⁺ DCs in the tumor stroma (Fig. 4). Consequently, we observed a trend toward a higher CD1a⁺/DC-LAMP⁺ cell ratio within the tumor epithelium and a significantly higher CD1a⁺/DC-LAMP⁺ cell ratio within the tumor stroma in relapsed patients with KRAS mutations in codon 13. Similar differences between disease-free and relapsed patients were observed in the entire cohort regardless of the KRAS mutational status (Fig. 5). However, multivariate analysis revealed that only the CD1a/DC-LAMP ratio in the tumor epithelium, proportion of Ki-67⁺ tumor cells, and number of intraepithelial CD8⁺ cells were independent prognostic factors of recurrence.

4. Discussion

Carcinogenesis is caused by both external and internal factors, and most types of tumors require the concurrence of multiple steps. However, despite extensive research, the mechanisms that control cancer progression and recurrence have not been fully characterized. Consequently, there is a strong need to establish reliable prognostic markers for each type of cancer to properly address postsurgical patient follow-up and identify patients who would benefit from adjuvant therapy.

One of the recently discussed possible prognostic factors in CRC patients is the mutational status of the KRAS gene. It has been reported that KRAS mutations occur early in colorectal carcinogenesis, often before the development of polyps [3,17,18]. The percentage of CRC patients with mutations in KRAS varies from 30 to 50%, most likely depending on the different techniques used for detection [7–9]. In our cohort of patients, we identified KRAS mutations in 45.5% of tumors, including 65% in codon 12 and 35% in codon 13, which agrees with previous studies [9,10]. Missense mutations at these positions that result in the replacement of glycine with a different amino acid lead to decreased GTPase activity in RAS. Because GTP-bound RAS is involved in a wide range of cellular processes by regulating the activation of at least 10 downstream effector pathways, these mutations markedly affect the proliferation, differentiation, and survival of tumor cells [11].

Analyses focused on the prognostic significance of KRAS mutations in CRC patients have reported conflicting results. Whereas some studies have reported a correlation between a reduced survival rate and the presence of KRAS mutations [19–27], other studies have not confirmed this association [28–32]. Several reports have indicated that only specific KRAS mutations may have a strong predictive value. Indeed, KRAS mutations in codon 12 have been observed to be associated with the mucinous histotype [9,19], whereas codon 13 mutations have been demonstrated to correlate with a high S-phase fraction [9] and reduced patient survival rate [9,26]. Similarly, we observed that tumors with a mutation in codon 13 had a significantly higher proliferation rate and exhibited a higher risk of disease recurrence than WT tumors and tumors with a codon 12 mutation. Because the percentage of Ki67⁺ tumor cells was an independent prognostic factor according to the multivariate analysis, we suggest that tumor proliferation rate, which reflects tumor aggressivity, may be the proximate cause of higher risk of disease recurrence in CRC patients with KRAS mutations in codon 13.

In addition, we observed marked variability in the pattern of tumor-infiltrating immune cells within the group of patients with codon 13 mutations, regardless of the mutation type. Patients who experienced tumor recurrence had fewer lymphocytes in both the tumor epithelium and the stroma. Additionally, relapsed patients...
had a significantly higher CD1a⁺/DC-LAMP⁺ cell ratio than disease-free patients. These data are in accordance with the theory that immune surveillance plays an essential role in cancer development and progression [33]. Indeed, it has been convincingly demonstrated that high densities of TILs predict a favorable outcome in CRC patients [12–16]. Galon et al. reported that CRC patients with an increased expression of T helper 1 adaptive immunity–associated genes in the tumor tissue had the best prognosis. Moreover, the presence of such an immune response seemed to be an even stronger independent predictor than TNM classification [15]. The prognostic impact of DC subtypes in CRC is much less clear. Dadabayev et al. [34] demonstrated that higher densities of MHC class II-expressing cells in the tumor stroma were associated with shorter survival. Conversely, the presence of MHC class II⁺ cells in the tumor epithelium was correlated with improved survival. Nagorsen et al. [35] observed a better survival rate in cases with both high stromal and high epithelial DC infiltration.

In our study, the phenotype of the tumor-infiltrating DCs seemed to be crucial. Patients with disease recurrence had higher densities of CD1a⁺ cells and significantly lower densities of mature DC-LAMP⁺ DCs in the tumor stroma (Fig. 5). This effect was even more pronounced in the group of patients with KRAS mutation in codon 13 (Fig. 4). Thus, the activation status of DCs in relapsed patients seems to be skewed toward immature DCs. Suzuki et al. suggested that part of CD1a⁺ tumor-infiltrating DCs migrated to the cancer stroma, lost CD1a expression during maturation, and recruited T cells to form DC–lymphocyte clusters where T-cell activation occurred [36]. Similarly, we also observed aggregates of DC-LAMP⁺ DCs in the tumor stroma of CRC patients, as illustrated in Fig. 1. On the contrary to DC-LAMP⁺ DCs, CD1a⁺ cells were observed throughout the tumor tissue without forming any clusters.

We suggest that our data might elucidate the discrepancies in the analyses focused on the prognostic significance of KRAS mutations. Indeed, disease-free (>50 months) and relapsed patients (confirmed relapse <50 months from the date of surgery) with codon 13 mutations in our cohort were markedly different in terms of their levels of tumor-infiltrating immune cells. Thus, CRC patients with low levels of TILs, a high CD1a⁺/DC-LAMP⁺ tumor-infiltrating DC ratio, and KRAS mutations in codon 13 might be at high risk of disease recurrence. Moreover, because these patients cannot benefit from anti-EGFR therapy, they might also be at high risk of cancer-related death. Because the quantification of immune responses within the tumors indicated a strong predictive role in...
CRC patients, the combined characterization of genetic features and immune cells might provide the foundation to identify the group of high-risk patients who would potentially benefit from adjuvant chemotherapy and careful follow-up.

Acknowledgments

We thank Vit Budinský for technical help with fluorescence microscopy. This project was supported by Research Grant MSM 0021620812 from the Czech Ministry of Education and GAUK 9939/2009 from Charles University.

References


