Concordance of KRAS mutation status between luminal and peripheral regions of primary colorectal cancer. A laser-capture microdissection-based study

Magdalena Lewandowska, Jolanta Hybiak, Wenancjusz Domagala

The presence of KRAS mutation in colorectal cancer (CRC) is a marker of resistance to anti-EGFR therapy. However, there are conflicting reports concerning intratumoral heterogeneity of KRAS mutations. The aim of this study was to determine whether within primary CRCs with KRAS mutations intratumoral KRAS mutation heterogeneity can be detected between two strictly defined areas, i.e. the luminal (mucosa/submucosa) and peripheral invasive front of the tumor. Using laser-capture microdissection, from every tumor about 400-500 nests of cancer cells were excised from each of the examined areas (luminal and peripheral) and PNACLamp, a high-sensitivity real-time PCR-based diagnostic assay for KRAS mutation testing, was used for molecular analysis. KRAS mutations were detected in codon 12 in both luminal and peripheral regions in all tumors examined. We conclude that from the point of view of practical KRAS mutation testing for predictive purposes in patients with CRC (i.e. testing mutations in codons 12 and 13) sampling errors are unlikely to occur if in CRCs with KRAS mutations only the luminal (as in biopsy tissue) or peripheral region is examined, provided a sensitive system of detection is applied and an appropriate number of tumor cells with minimal contamination by benign cells is analyzed.

Key words: KRAS mutation, colorectal cancer, laser microdissection, KRAS intratumoral homogeneity.

Introduction

KRAS mutation prevalence in patients with metastatic colorectal cancer (mCRC) is about 50% [11] and the vast majority appear in codons 12 (approximately 77% of mutations) and 13 (approximately 20%) in exon 2 of the gene [2]. Contemporary targeted therapy in mCRC utilizes antibodies directed against epidermal growth factor receptor (EGFR), e.g. panitumumab. However, despite promising results of early clinical trials it has become apparent that patients with activating KRAS mutations will not benefit from treatment with anti-EGFR antibody. Therefore KRAS mutation status has emerged as an important predictive marker for anti-EGFR therapy in patients with mCRC (for review see Domagala P. et al. [3]). However, 40% to 60% of patients with wild-type KRAS fail to respond to the treatment [4]. Among many possible hypotheses that could explain this phenomenon is the intratumoral heterogeneity of KRAS status, which might lead to false negative test results. KRAS mutation is regarded as an early event in multistep colonic carcinogenesis [5], so one would expect KRAS mutation homogeneity throughout a primary CRC, and indeed KRAS mutation homogeneity in primary CRCs has been reported [6, 7, 8, 9]. However, results of several studies suggest the presence of KRAS status heterogeneity in primary CRCs [10,
The conflicting results may be due to various molecular techniques used for detection of KRAS mutations in formalin-fixed paraffin-embedded (FFPE) tumor samples that are characterized by different specificities, sensitivities and complexities. For example, commercially available high-sensitivity real-time PCR-based diagnostic assays for KRAS mutation testing, with the CE-IVD mark, seem to be a better alternative to direct sequencing of FFPE tissue [3]. Tumor cellularity is also a critical issue, so low numbers of tumor cells obtained e.g. by macrodissection and contamination by benign cells may also play a role.

The aim of this study was to determine whether within primary CRCs with KRAS mutations any intratumoral heterogeneity of KRAS mutation status can be detected between two strictly defined areas, i.e. the luminal (mucosa/submucosa) and peripheral invasive front of the tumor. To this end, laser-capture microdissection was applied, because it is the most accurate technique to separate tumor cells from benign stromal cells, and a commercially available high-sensitivity real-time PCR-based diagnostic assay for KRAS mutation testing, with the CE-IVD mark, was used for molecular analysis.

**Material and methods**

The study was based on tumor tissue from 14 FFPE CRCs (from the archive of the Department of Pathology of Pomeranian Medical University) in which KRAS mutations had been previously detected with the PNAClamp KRAS mutation detection kit (Panagene, Daejeon, Korea) using manual microdissection. Details of clinicopathological characteristics of the study group are presented in Table I. Altogether there were one G1, 11 G2 and two G3 adenocarcinomas.

**Laser microdissection**

On the basis of ability to unambiguously determine the level of infiltration, one or two paraffin blocks containing tumor tissue fixed in 10% neutral buffered formalin were selected from each case, and 5-μm-thick sections were cut and placed onto membrane slides (Zeiss, Germany). The sections were stained with 0.1% (w/v) Cresyl Violet acetate (Sigma, Saint Louis, USA) in 50% alcohol. To obtain samples from two areas of the tumor, i.e. the luminal (mucosa/submucosa) and peripheral invasive border, a laser-capture microdissection system (PALM MicroBeam, Zeiss) was used. From every tumor about 400-500 nests of cancer cells were excised from each of the examined areas (i.e. luminal and peripheral) for molecular analysis. The nests of cancer cells were dissected without any intervening stroma, necrosis or inflammatory cells in order to enrich the tumor cell-derived DNA and were catapulted into adhesive caps. Representative images of CRC before and after laser microdissection are presented in Figs. 1 and 2.

**Table I. Clinicopathological characteristics of the study group and results of KRAS testing**

<table>
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<tr>
<th>NO.</th>
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<th>AGE</th>
<th>LOCATION</th>
<th>HISTOLOGICAL TYPE</th>
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<th>ASTLER-COLLER</th>
<th>KRAS L CODON</th>
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<td>B2</td>
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<td>cecum</td>
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L – luminal region (mucosa and submucosa)
P – peripheral invasive front
* < 50% mucinous carcinoma
Detection of \textit{KRAS} mutations

In total the DNA from 28 samples (14 from the luminal and 14 from the peripheral areas) was isolated with the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) and the amount of DNA was measured with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Wilmington, USA). Afterwards the presence of \textit{KRAS} mutations was detected with the PNAClamp \textit{KRAS} mutation detection kit (Panagene, Daejeon, Korea). Accuracy of results of \textit{KRAS} testing in the laboratory was validated by successfully passing the ESP \textit{KRAS} EQA scheme.
Results

PNAClamp detects 6 KRAS mutations in codon 12 (G12D c.35G>A, G12A c.35G>C, G12V c.35G>T, G12S c.34G>A, G12R c.34G>C, G12C c.34G>T) and one mutation in codon 13 (G13D c.38G>A). In each of 14 CRCs KRAS mutations were detected in codon 12 (Table I) in tumor tissue obtained from both regions, i.e. from the luminal area and the peripheral invasive front of the CRC. Thus, in respect of KRAS mutations detected by PNAClamp there were no differences between tumor tissue acquired from the two different areas examined (luminal and peripheral). Tumor stage or site did not influence the results.

Discussion

KRAS testing has become a routine test necessary to provide predictive information in pathology reports of CRC (for review see Domagala P. et al. [3]). High-sensitivity KRAS detection methods improve the prediction of benefit from targeted therapy, thereby justifying their use for routine KRAS testing. In this study we investigated the intratumoral distribution of KRAS mutations in primary CRCs using tumor samples obtained by laser-capture microdissection from two strictly defined areas of primary invasive CRC, i.e. the luminal and peripheral invasive front of the tumor. For molecular analyses the PNAClamp KRAS Mutation Detection Kit – a CE-IVD-marked high-sensitivity KRAS detection method – was used [in vitro diagnostic (IVD) assays in EU member countries are required to conform to IVD Directive requirements and to be CE-IVD marked [3]. The PNAClamp test is a very sensitive method for detecting mutations in a minute amount of DNA (optimal range: 10-25 ng of total DNA), and it is suitable for KRAS mutation testing in small biopsy specimens [15, 16]. In the present study intratumoral homogeneity of KRAS mutation distribution between luminal and peripheral parts of CRCs was detected, supporting the view that KRAS mutations occur early in the course of colorectal carcinogenesis [5] and remain during tumor progression to a more advanced stage.

There are only two reports dealing with KRAS mutation testing based on laser microdissection of CRCs. Bösmüller et al. [17] reported different activating KRAS mutations (in one patient) or a mutated and a non-mutated portion of a tumor (in another patient), but they were detected in morphologically distinct tumor components. Boub et al. [18] using laser microdissection and direct sequencing examined three CRCs positive for KRAS mutation. In one of these CRCs, KRAS mutation in the luminal zone was not confirmed in one of the three samples taken from the invasion front of CRC. However, the sensitivity of direct sequencing is regarded to be too low to be used for the analysis of KRAS mutations in FFPE tumor samples as a routine clinical test [3]. In our study we obtained multiple tumor samples (hundreds) by laser-capture microdissection and used the sensitive PNAClamp KRAS mutation detection system, and our findings suggest that if the KRAS mutation is present in primary CRC it occurs in tumor cells located in both luminal and peripheral regions of the tumor.

Our data support the results of Farber et al. [6], who, using manual microdissection and real-time PCR, evaluated the relative fraction of mutated versus wild-type KRAS alleles in FFPE CRCs carrying the KRAS mutation. In almost all (41 out of 42) tumors, the fraction of mutation containing tumor cells was 50% or higher, indicating the absence of significant KRAS mutation status heterogeneity. Ishii et al. [9] analyzed KRAS gene mutations in 21 CRCs using a crypt isolation technique, PCR and direct sequencing. They concluded that most CRCs did not show KRAS mutation heterogeneity and suggested that this may be the result of progression of one tumor clone with a KRAS mutation. Similar results and conclusion were obtained by Shibata et al. [7], who evaluated distribution of KRAS mutations in multiple areas of 7 primary CRCs and found KRAS mutations in all cancer cells studied. Also Dix et al. [8], who examined 11 primary CRCs at four different sites within the tumor, did not find KRAS mutation heterogeneity. The reports cited above suggest KRAS mutation homogeneity in primary CRC; however, the exact regions from which tumor samples were obtained for the analyses have not been reported. High concordance between the presence of KRAS mutations in primary CRCs and their respective liver or lung metastases also indirectly suggests KRAS mutation homogeneity in a primary tumor [19, 20].

However, results of other studies suggest KRAS status heterogeneity between primary CRC and blood-borne or lymph node metastases or KRAS mutation heterogeneity within the primary tumor. In respect of intratumoral heterogeneity Al-Mulla et al. [13] examined 78 primary CRCs for KRAS mutations in codons 12 and 13. Tissue samples for allele-specific oligonucleotide hybridization and sequencing were obtained with microneedles using a Leitz model M micromanipulator. Multiple primary tumor sampling revealed KRAS mutation heterogeneity (in 9 of 26 primary tumors with KRAS mutations there were also areas of carcinoma with only the wild-type gene), but the exact location of the mutated and wild type areas within the tumor was not provided. Losi et al. [10] suggest that whereas heterogeneity of KRAS mutations may be found in early CRC (mucosa/submucosa), in advanced cases it may be lost due to the presence of
a predominant clone. On the other hand, Fukunari et al. [12] reported that intratumoral KRAS mutation heterogeneity (i.e. two or more regions of a CRC with variations of genetic changes) appeared to be more common in advanced disease than in CRCs without metastases. Oltedal et al. [21] examined the primary tumors of patients with discordance of KRAS status between fresh-frozen samples of the outer rim of primary tumors and the lymph node metastasis. In 10 CRCs KRAS mutations were found in some FFPE blocks and were not detected in others, and in 5 cases mutation was detected in lymph node metastases, even though no KRAS mutation was detected in the primary tumor. Baldus et al. [14] examined intratumoral heterogeneity between the tumor center and corresponding invasion front of 100 CRCs. Tumor tissue was obtained by macrodissection of unstained sections. The KRAS mutations were revealed by two methods: cycle sequencing analysis of PCR and pyrosequencing. In 8% of CRCs intratumoral heterogeneity was detected, including 6% of tumors in which a mutation was found in the tumor center but not at the corresponding invasion front and 2% of tumors with an inverse pattern. Giaretti et al. [11] examined multiple samples taken by macrodissection from superficial and deep parts of 9 frozen CRCs with KRAS mutations. Intratumoral homogeneity of the KRAS mutation was detected in two-thirds of the cases and intratumoral heterogeneity in the remaining one-third. Knijn et al. [19] reported two CRCs showing heterogeneity of KRAS status within the primary tumor. Unfortunately the exact regions from which tissue was taken for testing were not reported.

Thus, although intratumoral heterogeneity of KRAS mutations in CRCs has been reported, in these reports mechanical microdissection [22] or macroscopic dissection [11, 12, 14, 23] was applied, so the results may be due to low numbers of acquired tumor cells and benign cell contamination. Furthermore, the discrepancies may, at least partially, be attributed to low sensitivity methods of KRAS mutation detection and fresh frozen vs. FFPE tissue used in various studies. For example, Bando et al. [23] compared the evaluation of KRAS status in 159 macrodissected FFPE CRCs by direct sequencing and by an amplification refractory mutation system – Scorpion assay (ARMS/S). All mutations identified by direct sequencing were also identified by ARMS/S. However, 7.0% of the 70 KRAS mutations identified by ARMS/S were not detected by direct sequencing. In another study Malapelle et al. [24] showed that high resolution melting analysis (HRMA) identified mutations in 4/50 patients previously found to be KRAS-wild type by direct sequencing. Aldorf et al. [25] analyzed 3-8 different tumor areas of 40 non-small cell lung cancers and in 4 cases found heterogeneous KRAS results by direct sequencing. However, when they applied a more sensitive method (laser-capture microdissection for tumor cell enrichment and ARMS/S method), KRAS mutation analysis revealed that in these 4 tumors the results were false negative due to the admixture of non-neoplastic cells in all samples.

In conclusion, using laser-capture microdissection and the PNAClamp KRAS mutation detection system no KRAS mutation heterogeneity was found between luminal and peripheral invasive parts of CRCs with KRAS mutations irrespective of tumor grade and site. This supports the idea that KRAS mutations are acquired early and remain during progression of CRC. Our results suggest that from the point of view of practical KRAS mutation testing for predictive purposes in patients with mCRC (i.e. testing mutations in codons 12 and 13) the region of CRC from which tumor tissue is microdissected should not significantly influence the results in cases with KRAS mutations, provided a sensitive system of detection is applied and an appropriate number of tumor cells with minimal contamination by benign cells is analyzed. The results also suggest that sampling errors are unlikely to occur in CRCs with KRAS mutations if only the luminal (as in biopsy tissue) or peripheral invasive region of CRC with KRAS mutations is supplied for molecular analysis.

The authors declare no conflict of interest.

References


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