Original paper

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

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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% [1] 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,

11, 12, 13, 14]. 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

No.	Sex	Age	Location	Histological type	pTNM	Astler-Coller	KRAS L codon	KRAS P codon
1	M	60	rectum	adenocarcinoma G2	T3NxMx	B2/C2	12	12
2	M	78	no data	adenocarcinoma G2a	T3N0Mx	B2	12	12
3	F	56	cecum	adenocarcinoma G3	T3N2Mx	C2	12	12
4	F	65	rectum	adenocarcinoma G2	T3N2Mx	C2	12	12
5	F	65	cecum	adenocarcinoma G2	T4aN2bM1b	D	12	12
6	M	66	no data	adenocarcinoma G2	no data	no data	12	12
7	M	61	splenic flexure	adenocarcinoma G1	T3N0Mx	B2	12	12
8	F	76	rectum	adenocarcinoma G2	no data	D	12	12
9	F	71	sigmoid colon	adenocarcinoma G2	T3N2Mx	C2	12	12
10	F	73	ascending colon	adenocarcinoma G2[a]	T3N1/2M1	D	12	12
11	M	73	hepatic flexure	adenocarcinoma tubulopapillary G2[a]	T3N2Mx	C2	12	12
12	M	64	ascending colon	adenocarcinoma G2	T3N1/2Mx	C2	12	12
13	M	72	cecum	adenocarcinoma G2	T3N0Mx	B2	12	12
14	M	64	no data	adenocarcinoma G3	T3N2M1	D	12	12

L – luminal region (mucosa and submucosa)
P – peripheral invasive front
[a] *< 50% mucinous carcinoma*

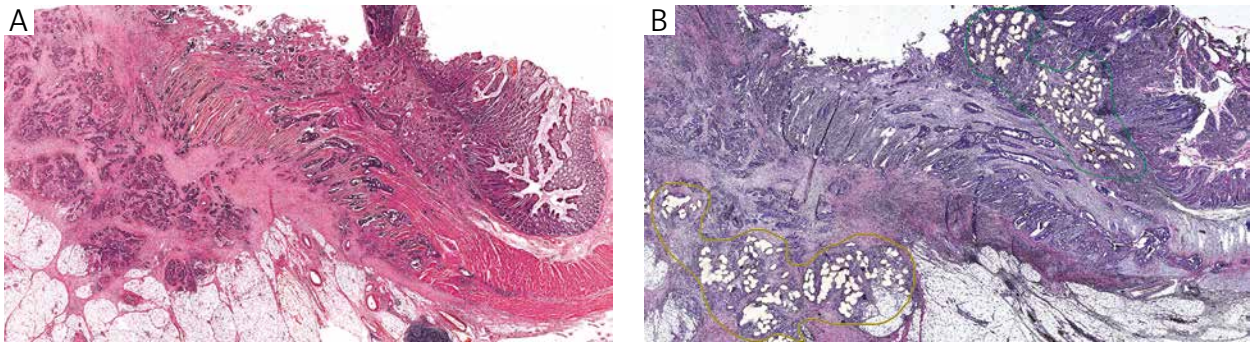

Fig. 1. A) Colorectal cancer invading colon from mucosa/submucosa to peripheral fat. HE stain. B) Laser-capture microdissection. Overview of microdissected (empty spaces) luminal (green circle) and peripheral (yellow circle) regions of the tumor in Fig. 1A

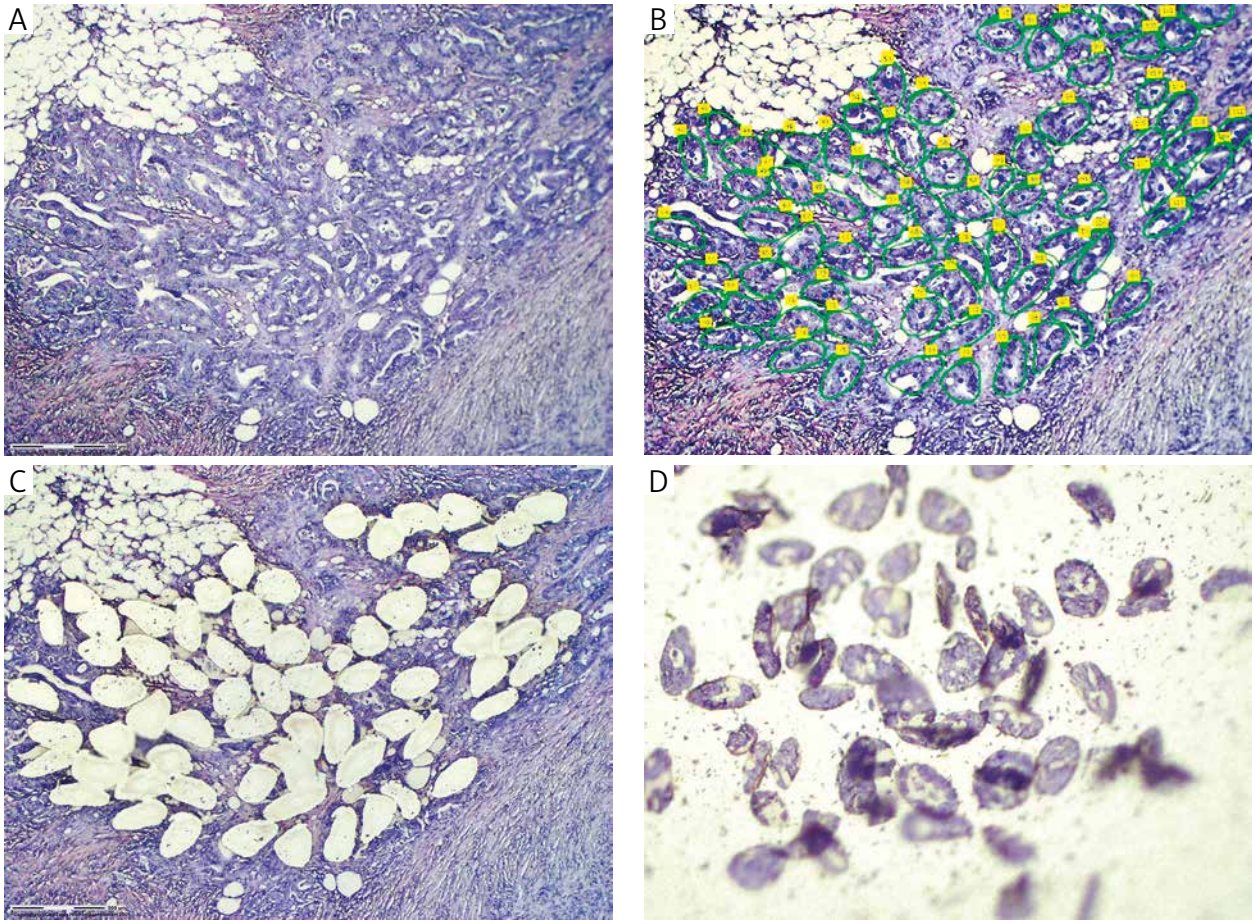

Fig. 2. Four steps of laser-capture microdissection. A) Nests of cancer cells before microdissection. B) Nests of tumor cells marked for microdissection. C) Empty spaces left after tumor cells had been dissected. D) Microdissected tumor cells catapulted into an adhesive cap

Detection of *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 *KRAS* mutations was detected with the PNAClamp *KRAS* mutation detection kit (Panagene, Daejeon, Korea). Accuracy of results of *KRAS* testing in the laboratory was validated by successfully passing the ESP *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 mutants 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. Alsdorf *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

1. Peeters M, Kafatos G, Taylor A, et al. Prevalence of RAS mutations and individual variation patterns among patients with metastatic colorectal cancer: A pooled analysis of randomised controlled trials. Eur J Cancer 2015; 51: 1704-1713.
2. Smith G, Bounds R, Wolf H, et al. Activating K-Ras mutations outwith 'hotspot' codons in sporadic colorectal tumours – implications for personalised cancer medicine. Br J Cancer 2010; 102: 693-703.
3. Domagala P, Hybiak J, Sulzyc-Bielicka V, et al. KRAS mutation testing in colorectal cancer as an example of the pathologist's role in personalized targeted therapy: A practical approach. Pol J Pathol 2012; 63: 145-164.
4. Linardou H, Dahabreh IJ, Kanaloupiti D, et al. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol 2008; 9: 962-972.
5. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61: 759-767.
6. Farber L, Efrati E, Elkin H, et al. Molecular morphometric analysis shows relative intra-tumoural homogeneity for KRAS mutations in colorectal cancer. Virchows Archiv 2011; 459: 487-493.
7. Shibata D, Schaeffer J, Li ZH, et al. Genetic-heterogeneity of the c-k-ras locus in colorectal adenomas but not in adenocarcinomas. J Nat Cancer Inst 1993; 85: 1058-1063.
8. Dix BR, Robbins PD, Spagnolo DV, et al. Clonal analysis of colorectal tumors using k-ras and p53 gene-mutations as markers. Diagn Molec Pathol 1995; 4: 261-265.

9. Ishii M, Sugai T, Habano W, Nakamura S. Analysis of Ki-ras gene mutations within the same tumor using a single tumor crypt in colorectal carcinomas. J Gastroenterol 2004; 39: 544-549.
10. Losi L, Baisse B, Bouzourene H, Benhattar J. Evolution of intratumoral genetic heterogeneity during colorectal cancer progression. Carcinogenesis 2005; 26: 916-922.
11. Giaretti W, Monaco R, Pujic N, et al. Intratumor heterogeneity of K-ras2 mutations in colorectal adenocarcinomas. Am J Pathol 1996; 149: 237-245.
12. Fukunari H, Iwama T, Sugihara K, Miyaki M. Intratumoral heterogeneity of genetic changes in primary colorectal carcinomas with metastasis. Surg Today 2003; 33: 408-413.
13. Al-Mulla F, Going JJ, Sowden E, et al. Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. J Pathol 1998; 185: 130-138.
14. Baldus SE, Schaefer K-L, Engers R, et al. Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. Clin Cancer Res 2010; 16: 790-799.
15. Nagai Y, Miyazawa H, Huqun, et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res 2005; 65: 7276-7282.
16. Jeong D, Jeong Y, Lee J, et al. Rapid and sensitive detection of KRAS mutation by peptide nucleic acid-based real-time PCR clamping: A Comparison with direct sequencing between fresh tissue and formalin-fixed and paraffin embedded tissue of colorectal cancer. Kor J Pathol 2011; 45: 151-159.
17. Bösmüller H, Kranewitter W, Webersinke G, et al. Morphological and molecular heterogeneity in colorectal neoplasms with K-RAS mutation. A report of two cases. Pathol Res Pract 2011; 207: 399-402.
18. Buob D, Fauvel H, Buisine MP, et al. The complex intratumoral heterogeneity of colon cancer highlighted by laser microdissection. Dig Dis Sci 2012; 57: 1271-1280.
19. Knijn N, Mekenkamp LJM, Klomp M, et al. KRAS mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer patients. Br J Cancer 2011; 104: 1020-1026.
20. Cejas P, López-Gómez M, Aguayo C, et al. KRAS mutations in primary colorectal cancer tumors and related metastases: A potential role in prediction of lung metastasis. PLoS ONE 2009; 4: e8199, doi: 10.1371/journal.pone.0008199.
21. Oltedal S, Aasprong OG, Moller JH, et al. Heterogeneous distribution of K-ras mutations in primary colon carcinomas: implications for EGFR-directed therapy. Int J Colorectal Dis 2011; 26: 1271-1277.
22. Baisse B, Bouzourene H, Saraga EP, et al. Intratumor genetic heterogeneity in advanced human colorectal adenocarcinoma. Int J Cancer 2001; 93: 346-352.
23. Bando H, Yoshino T, Tsuchihara K, et al. KRAS mutations detected by the amplification refractory mutation system-Scorpion assays strongly correlate with therapeutic effect of cetuximab. Br J Cancer 2011; 105: 403-406.
24. Malapelle U, Carlomagno C, Salatiello M, et al. KRAS mutation detection by high-resolution melting analysis significantly predicts clinical benefit of cetuximab in metastatic colorectal cancer. Br J Cancer 2012; 107: 626-631.
25. Alsdorf WH, Clauditz TS, Hoenig T, et al. Intratumoral heterogeneity of KRAS mutation is rare in non-small-cell lung cancer. Exper Molec Pathol 2013; 94: 155-159.

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