Abstract. Real-time PCR-based molecular diagnostic techniques were used for the comparative identification of the most common KRAS and BRAF mutations from methanol liquid-based cytology samples and matched, formalin-fixed and paraffin-embedded tissue samples. Methanol-based fixation and preservation of brushing samples of colorectal carcinomas could contribute to sample collection, short-term storage and subsequent molecular analysis, by improving workflow and enhancing the identification of putative mutations.

Colorectal carcinoma is one of the most common types of cancer worldwide (1). Despite the wide range of therapeutic approaches currently used, morbidity and mortality remain high, especially in advanced-stage patients (2, 3). Regimens targeting EGFR constitute one of the most promising novel therapeutic strategies (4). The absence of activating KRAS mutations reducing or abolishing EGFR inhibition is the necessary pre-requisite for such a treatment (5, 6).

KRAS is a protein kinase acting downstream of EGFR, commonly mutated in many carcinomas (7). The activating mutation at codon 600 (V600E) of BRAF, another molecule involved in the same signaling cascade, is frequently found in melanomas, as well as in various carcinomas (8). Although one might theoretically expect that activating BRAF mutations would have the same effect in anti-EGFR therapy, there are, so far, limited clinical data in support of this assumption (9, 10).

Even though colorectal carcinoma is diagnosed only by histological examination of biopsies or surgical specimens, cytology of the colorectal tract is currently evaluated for screening or diagnosing colorectal lesions (11). Formalin fixation and paraffin embedding are the mainstream procedures for histological samples, but the quality of DNA retrieved from these samples may be suboptimal, resulting in variability of DNA extraction efficiency and quality (12, 13). However, methanol and fixatives used in ThinPrep® (Hologic, Bedford, USA) liquid-based cytology are considered to be excellent nucleic acid preservatives (13, 14).

The aim of this study was to examine differences in mutation analysis between formalin-fixed, paraffin-embedded (FFPE) samples and those used for liquid-based cytology.

Materials and Methods

Sixty four biopsy-diagnosed carcinomas from surgical specimens received consecutively in the Second Department of Pathology within a period of 4 months from January until April of 2009 were included in this study. Basic epidemiological and clinical data (age, sex, tumour site) were collected and the classic pTNM classification (15) was retrieved from the histology reports.

Sample collection and DNA extraction. Following macroscopic identification of the tumour in the resection specimen, its surface was twice brushed with a commercially available, sterile cytological brush (EndoBrush, Biogyn, Mirandola, Italy) and collected cells were stored in Cytolyt® (Hologic, Marlborough, USA) until DNA extraction (mean storage time 8-48 hours at 4°C). Moreover, a small tumour fragment was snap-frozen at −80°C. The colectomy specimen was thereafter immersed in a 10% buffered formal fixative solution, dehydrated and paraffin embedded in an automated tissue
DNA extraction was performed with Purelink™ DNA Mini KIT (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions. For ThinPrep® brushings and frozen tumour specimens, DNA extraction was washed twice with PBS prior to proteinase K digestion.

**DNA quantification.** Extracted DNA concentration and yield was determined by fluorometry, with QuantIt™ PicoGreen® Assay kit (Invitrogen). Briefly, serial dilutions of 100 to 1 ng/μl were used to create a standard curve. Five μl of an appropriate dilution of sample DNA was added to a 45 μl reaction containing diluted PicoGreen® (Invitrogen) dye.

**Real-time PCR.** Detection of KRAS mutations of codons 12 and 13 was performed with a commercially available Real-Time PCR kit (Therascreen KRAS, DxS Diagnostics, Manchester, UK) detecting 6 mutations of codon 12 (G12D, G12A, G12V, G12S, G12R, G12C) and 1 mutation of codon 13 (G13D). A positive reaction mix for all mutations was included (Figure 1a); moreover, a second exogenous reaction was simultaneously taking place, to avoid false-negative results caused by PCR inhibitors (Figure 1b). Samples were characterized as bearing a mutation only if ΔCt (Ct of control reaction – Ct mutation reaction) was lower than the value set by the manufacturer. The control reaction amplifies a part of exon 4 of KRAS.

Activating mutation V600E of BRAF was identified using molecular beacons. After thermal characterization of the beacons (Figures 2a, 2b), one beacon for the wild-type and one for the mutant allele (Table I) were added at a final concentration of 100 nM in a 25 μl PCR reaction containing 1xPCR buffer, 6 mM MgCl₂, 200 nM dNTPs, 300 nM of each primer (Table I) and 1U of Platinum® Taq (Invitrogen). PCR profile used was 95°C 2 min., followed by 40 cycles of 95°C for 10 s, 62°C for 60 s and 72°C for 20 s. Fluorescence was measured during the annealing step. Positive control used for both the wild-type and mutant allele was a clinical sample previously identified as carrying the mutation with sequencing. all real-time PCR experiments were performed on a Corbett 6000 instrument (Corbett Life Sciences, Australia).

**Sequencing.** Part of exon 2 of KRAS from selected samples was amplified with a previously published primer set (Table I). Fifty ng of extracted DNA was added to a PCR reaction containing 1xPCR buffer, 1.5 mM MgCl₂, 200 nM dNTPs, 150 nM of each primer and 1U of Platinum® Taq. The PCR profile used included an initial denaturation step at 94°C for 2 min, 35 cycles consisting of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by a final extension step at 72°C for 3 min. Exon 15 of BRAF was amplified with a primer set designed in order to avoid a BRAF pseudogene located at chromosome 7. PCR reaction setup and profile were identical with those used for KRAS. PCR products were analyzed on 2% w/v agarose gels, stained with ethidium bromide and visualized on a GeneGenius system (SynGene, Cambridge, UK). Bands were excised from gel and PCR products were purified with Purelink™ Quick Gel Extraction KIT (Invitrogen). Sequencing was performed at Lark (Lark Technologies, Essex, UK). Chromatographs were analysed with FinchTV (Geospiza, Seattle, USA) for abnormalities.

**Primers and beacons.** All previously unpublished primers and molecular beacons were designed with Beacon Designer 7 (Premier Biosoft, Palo Alto, USA) (Table I). Oligos were synthesized by Jena Bioscience GmbH (Jena, Germany).

**Statistical analysis.** Statistical analysis of the results was performed using Fisher’s exact test, Student’s t-test using two-tailed paired analysis and linear regression analysis.

**Results**

Epidemiological, clinical and pathological data of patients included in this study, as well as results concerning the status of genes investigated are shown in Table II. DNA extraction quantification revealed that ThinPrep® brushings had significantly higher average concentration (62.1 vs. 25.84 ng/μl, p=0.018) and total yield (6.2 μg vs. 1.2 μg) compared to FFPE specimens. For the 32 wild-type KRAS samples, no difference in genotype was observed between specimens with either fixation. DNA isolated from ThinPrep® had a significant lower Ct value (26.09 vs. 28.15, p<0.001), which was inconsistent with the use of similar amount of DNA for amplification (Figure 3). During amplification of DNA isolated from paraffin samples, a wild-type genotype was erroneously identified in five cases, shown (in cytological specimens) as carrying a mutation. Two out of these five samples bore a G12C mutation, but the ΔCt was lower than that proposed by the manufacturer; repeating DNA isolation with thicker (10 μm) sections from the paraffin block resolved the issue, since the ΔCt fell in range (Figure 4a). However, the other three samples (1 G12S and 2 G13D) were still recognized as wild-type, even with the use of thicker sections. In order to verify the initial mutation found in ThinPrep®, DNA extraction from the frozen tissue sample stored at −80°C was carried out. Only then did it become feasible to detect the mutations (Figure 4b).

In general, ThinPrep® DNA had a lower Ct (27.1 vs. 28.9 p=0.005) and ΔCt (3.1 vs. 4.5, p=0.004) for the mutation reaction as compared with FFPE DNA (Figure 3). Furthermore, the exogenous control reaction for ThinPrep® failed only in three samples, which had to be re-amplified. On the other hand, nine FFPE DNA samples failed on one or more exogenous control reactions, probably indicating the presence of inhibitors. All types of mutations were verified with direct sequencing of exon 2 of KRAS. For samples bearing mutations with a ΔCt of over 4, the chromatogram could be misinterpreted as wild-type, since the second peak could be mistaken for background fluorescence (Figure 4c).

In our study, KRAS mutations were more frequently found in samples from the right colon (69.5% vs. 39.0%, 16/23 vs. 16/41, p=0.036) compared to those from the left
colon, consistent with other investigators’ findings (16). However the limited number of our samples cannot provide any further biological or clinical explanation for this finding.

The BRAF V600E mutation was identified in three samples (4.68%), all of which were located in the right colon and contained wild-type KRAS (Figures 5a, 5b). Of these cases, one was a female aged under 50, suspected, on the basis of pre-operative clinical assessment, to be a HNPCC patient (17) and identification of the mutation excluded her from the HNPCC group (18). Due to the small number of positive samples, no statistical difference between ThinPrep® and FFPE was found.

Figure 1. KRAS Scorpion reaction curves. Real-time PCR graphs depicting: a) green fluorescence channel curves for the positive reaction mix and for the no-template control (NTC, dH2O), only the positive control reactions have an increase of fluorescence; and b) red fluorescence channel curves for the same samples. Both types of samples are positive for the exogenous reaction control, used to verify absence of PCR inhibitors. Ctl: Control, NTC: no-template control.

Figure 2. Molecular Beacons thermal characterization. Thermal characterization of molecular beacons was performed by incubating the beacons with a perfect complementary oligonucleotide and measuring fluorescence while decreasing temperature in stepped intervals. Curves are from reactions that contain either a complementary sequence to the mutant type (red curves), or to the wild-type (green curves). Black curves do not include any complementary sequence. Fluorescence increased when the beacon hybridized to its complementary. a) Green channel curve (mutant beacon). b) Red channel (wild-type beacon). Both beacons’ fluorescence increased when they hybridized to their perfect match. Fluorescence due to hybridization to a mismatched sequence occurred at significantly lower temperatures, allowing the discrimination of the alleles.

Figure 3. Ct and ΔCt values of Scorpion and Molecular Beacon reactions.
Two out of three BRAF V600E mutated samples were verified with direct sequencing of the exon 15 PCR product, whereas the last was identified as wild-type (Figure 5c). The last sample had a ΔCt of over 6.

**Discussion**

Recent studies have underlined the importance of targeted therapy, especially in selecting cancer patients who can benefit from specific chemotherapeutic protocols (2). As a result, there is a growing need for sensitive, fast and reliable techniques to identify molecular changes, which can have an effect in patient selection. Thus, both the technique and the specimen type are of paramount importance.

In this study, Real-Time PCR was used for the detection of activating mutations of KRAS and BRAF, since their absence makes the patient a candidate for therapies that target EGFR (5, 6, 10). Real-time PCR was chosen for two main reasons: first, because it is a very sensitive and time-saving technique, and second, because all activating mutations examined are compacted in four DNA bases of KRAS and one base of BRAF. As a result, a fairly accurate analysis can be performed, since over 90% of KRAS and 80% of BRAF mutations are located in these three codons (8, 19).

In order to be able to identify low copy number mutations of KRAS, a commercial kit combining the amplification refractory mutation system technology (ARMS®, AstraZeneca, Cheshire, UK) with Scorpion® primers (DxS Ltd, Manchester, UK), both of which are ideal for mutation analysis, was used (20, 21). Additionally, the activating mutation V600E of BRAF was identified with the use of molecular beacons, due to their unique ability to easily discriminate single substitutions (22). Overall, it was possible to evaluate the mutation status of 6 samples only two hours after DNA extraction, with almost identical results as those obtained with sequencing.

Despite the limited number of our specimens, these results agree with those already published concerning the increased frequency of KRAS and BRAF mutations in carcinomas of the right colon compared to those in the left colon (16).

A common drawback for genetic testing from clinical samples is sample heterogeneity: a variable percentage of cancer cells are included in most tissue blocks, with ‘contaminating’ tumour stroma cells reducing testing sensitivity. These drawbacks can be overcome with complex techniques, such as laser-capture microdissection, that are costly and time consuming. In this study, it is proposed that a simple sample brushing, followed by fixation and storage in a methanol based preservative, is equally efficient for increasing DNA extraction concentration, as well as for analysing the

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Table II. Epidemiological, clinical, pathologic and genetic characteristics of the population studied.

<table>
<thead>
<tr>
<th>Patient no. (%)</th>
<th>Age</th>
<th>Gender</th>
<th>Tumour site</th>
<th>TNM</th>
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<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Left colon</td>
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<tr>
<td></td>
<td></td>
<td>Female</td>
<td>Right colon</td>
<td>T2</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
<td>N1</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mx</td>
</tr>
<tr>
<td>KRAS Wild-type</td>
<td>32</td>
<td>50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>32</td>
<td>50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G12D</td>
<td>10</td>
<td>31%</td>
<td></td>
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<tr>
<td>G13D</td>
<td>9</td>
<td>28%</td>
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<tr>
<td>G12C</td>
<td>6</td>
<td>19%</td>
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<td>G12S</td>
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<td></td>
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<tr>
<td>G12V</td>
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<tr>
<td>BRAF Wild-type</td>
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<td>95%</td>
<td></td>
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</tr>
<tr>
<td>Mutated</td>
<td>3</td>
<td>5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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P=Primer, MB=molecular beacon, S= sequencing, RT-PCR= real time-PCR. Bases in italic depict the differences between the mutated and wild-type beacon. Underlined bases depict the stem sequence of the beacon.
Figure 4. KRAS mutation analysis. A sample carrying a G12C mutation identified using extracted DNA from ai) ThinPrep, aii) 5 μm FFPE sections and aiii) 10 μm FFPE sections. ΔCt values were 3.68, 8.18 and 6.78 respectively. A sample with a G12S mutation identified using extracted DNA from bi) ThinPrep (ΔCt 5.49), bii) FFPE and biii) fresh frozen tissue (ΔCt 4.29), where the mutation could not be identified from FFPE material. Sequencing of the PCR products verified the existence of the mutations (aiv, biv). Samples with low ΔCt were easily identified with sequencing (ci, cii), while chromatographs from samples with higher ΔCt could be misinterpreted as wild-type (ciii, civ).

Figure 5. BRAF V600E mutation. Samples carrying the V600E mutation fluorescent at the green channel (a), while wild-type samples fluorescent at the red channel (b). All samples bearing the mutation were also positive for the wild-type reaction, indicating that only a fraction of carcinoma cells carry the mutation, making the mutation difficult to be identified on sequencing chromatographs (ci, cii).
mutations of KRAS, by increasing sensitivity and reducing repeats. This could also hold true for BRAF; nevertheless, results obtained from the limited number of positive samples in this study need to be corroborated with larger series.

The high efficacy of cytological specimens for this genetic analysis may be due to the inherent nature of sample collection. A large percentage of malignant cells may be obtained in brushings from the surface of both fungating and ulcerative colonic carcinomas, since carcinoma cells tend to have looser junctions with one another. Furthermore, DNA retrieved from formalin-fixed material has been shown to have reduced solubility and contain more PCR inhibitors, than that obtained from fresh frozen samples (13).

It can be argued that the difference observed in Ct and ΔCt values was due to differences in extracted DNA concentration. This was not the case here, since during statistical analysis, no correlation between ΔCt and any other parameter was found.

Furthermore, although samples containing ThinPrep® DNA showed lower ΔCts in general (3.1 vs. 4.5, p=0.004), especially for G12V, DNA retrieved from paraffin-embedded tissue had lower ΔCts (0.09 vs. 1.33), indicating that cells carrying the specific mutation may lay deeper within the tumour, thus limiting their availability in brushing samples (Figure 3).

The reduced time from bench to diagnosis when brushing specimens are used instead of FFPE samples (estimated at 2 days in the study Hospital) makes this technique of major practical importance both in the ever-increasing diagnostic burden of a modern pathology department and for colonoscopy samples coming from primary care units.

According to the results of this study, even though colorectal carcinoma brushings cannot be used to assess tumour stage or grade, they can be used for the identification of malignant cells, as well as for their accurate molecular characterization. The clinical relevance of these results is more evident in cases of unsectactable carcinomas, where the determination of the tumour molecular profile will assist the orchestration of neo-adjuvant treatment, in order to enhance the resectability of the tumour (23).

In conclusion, methanol containing liquid-based cytology fixatives, such as ThinPrep®, may contribute significantly to sample collection, short-term storage and subsequent molecular analysis of colorectal carcinomas, by improving workflow and enhancing mutation identification. In the era of personalized cancer remedies, molecular identification in cytological brushings must be further evaluated in order to be incorporated as a reliable, fast and safe tool in the armamentarium of modern cancer care.

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References


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