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Concordance in *KRAS* and *BRAF* mutations in endoscopic biopsy samples and resection specimens of colorectal adenocarcinoma

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Abstract ***Background:*** *KRAS* testing is mandatory if anti-*EGFR* therapy is considered in patients with metastatic colorectal cancer (CRC). In addition, *BRAF* mutations seem to be an important negative prognostic factor. The aim of this study is to establish the concordance of *KRAS* and *BRAF* mutational status in paired biopsy and resection specimens of primary CRC using several analytic methods.

Methods: DNA was extracted from paraffin blocks of 126 CRC patients. *KRAS* codon 12/13 and *BRAF* V600E mutational status was assessed using high resolution melting (HRM), direct sequencing (DS) of the HRM polymerase chain reaction (PCR) product. In addition, the Therascreen Amplification Refractory Mutation System (ARMS)-Scorpion *KRAS* assay and *BRAF* pyrosequencing were employed; both assays claim to require less tumour cells in comparison with DS.

Results: *KRAS* and *BRAF* were found to be mutually exclusive. Mutation frequencies were 33.9% for *KRAS*, and for *BRAF* 19.0%, respectively. Concordance of *KRAS* mutational status between biopsy and resection specimens was 97.4% (ARMS), 98.4% (DS) and 99.2% (HRM), respectively. For *BRAF* concordance was 98.4% (Pyro, DS) and 99.2% (HRM).

Conclusions: *KRAS* and *BRAF* mutational status of endoscopic biopsies and resection specimens of CRC showed a >95% concordance. Endoscopic biopsies can be confidently used for molecular analysis.

1. Introduction

The demand for *KRAS* mutational analysis as a predictive marker has increased rapidly. Prior to treatment with epidermal growth factor receptor (EGFR) inhibitors in colorectal cancer (CRC), *KRAS* testing has become mandatory in the European Union[1,2] and is

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recommended in the United States.[3] In addition, *BRAF* mutations are emerging as a very strong negative prognostic factor in CRC.[4]

KRAS is a member of the RAS proteins which are small GTPases that act as molecular switches. *KRAS* binds to Guanosine triphosphate (GTP) in the active state and has an intrinsic enzymatic activity which cleaves the terminal phosphate of the nucleotide converting it to Guanosine diphosphate (GDP).[5,6] Upon conversion of GTP to GDP, *KRAS* is turned off.[5,7] The *KRAS* protein has an important role in Ras/MAPK signalling in which GTP-bound *KRAS* regulates a variety of cellular processes, including proliferation, differentiation and apoptosis.[6,8] In CRC, codon 12 and 13 of the *KRAS* gene are mutated in 35% of cases,[9] transforming the intrinsic GTPase activity of the protein in the constitutively active conformation.[10]

BRAF, a member of the Raf kinase family of serine/threonine-specific protein kinases, is a cytosolic protein kinase and is activated by membrane-bound RAS. Mutated *BRAF* activates a signalling pathway, which causes cell proliferation and inhibits apoptosis.[10] The most common mutation is a single glutamic acid for valine substitution at codon 600 causing the V600E point mutation,[7] the *BRAF* mutation frequency is 11%.[9] Earlier, Roth and colleagues[11] described the mutual exclusivity of *BRAF* and *KRAS* mutations.

The activating mutations in *KRAS* and *BRAF* induce constitutive Ras/MAPK signalling, which cannot be suppressed by EGFR inhibition. Due to prevent undesirable side-effects of the EGFR antibodies and to suppress excessive treatment costs *KRAS* mutation analysis prior to anti-EGFR therapy is indicated.[12] In addition, *BRAF* mutational analysis yields prognostic information.[11]

Currently the most commonly used method for *KRAS* and *BRAF* mutation analysis is direct sequencing (DS). Nevertheless, this method requires high tumour cell percentages and good quality material. Resection specimens normally show large amounts of tumour cells. Biopsies generally consist of small tumour foci that might not meet the requirements for direct sequencing. An alternative is high-resolution-melting (HRM) analysis. High resolution melting (HRM) is advocated to be a rapid and cheap 'pre-screen' method, and can distinguish wild type from mutated genes based on subtle differences in temperature-depended denaturation ('melting') of double strand DNA fragments. This method requires considerably lower tumour cell percentages compared to DS but is unable to define the exact nature of a mutation when non-wild type melting curves are found. Other methods allowing low tumour cell percentages are pyrosequencing and a specific Amplification Refractory Mutation System (ARMS)-Scorpion PCR assay.

To date, no systematic study has been performed to ascertain the validity of *KRAS* and *BRAF* mutational analysis of endoscopic biopsy material in comparison to resection specimens.

The aim of our study was to establish the concordance of *KRAS* and *BRAF* mutational status between paired endoscopic biopsy and resection specimens in an unselected group of CRC patients. Three molecular assessment methods were used, e.g. DS and HRM for both genes, and in addition DxS ARMS-Scorpion Therascreen for *KRAS* and Qiagen Therascreen Pyro kit for *BRAF*.

2. Patients and methods

2.1. Patient selection

Colorectal adenocarcinoma cases were retrieved from the Department of Pathology, Isala klinieken, Zwolle, The Netherlands from the 2002 to 2005 period. Next, cases were selected: (i) colorectal endoscopic biopsies with an unequivocal report stating 'colorectal adenocarcinoma', and (ii) subsequent colorectal resection without any prior treatment stating unequivocally 'colorectal adenocarcinoma'. Archival formalin fixed paraffin-embedded (FFPE) tumour blocks were retrieved of both biopsy and resection specimens; 5 μm slides were cut and stained with haematoxylin & eosin (H&E); cases lacking adequate tumour tissue (defined as less than approx. 10% tumour percentage) were excluded. In total, 126 cases remained for the present study. Patient gender, age, location of CRC and stage were provided; right sided CRC was defined as: coecum, colon ascendens and colon transversum and left sided CRC as colon descendens and sigmoid. *KRAS* and *BRAF* mutational status was correlated with gender, age, CRC location and stage; statistical analysis was performed using Chi-square test for categorical variables or Fisher's in the case of dichotomous variables with small groups, all 2-tailed using alpha 0.05 as significance level. Analysis was performed using PASW version 18 (SPSS Inc., Chicago, IL, USA).

The Ethical Committee of the Isala klinieken declared that the study was not subjected to their approval being exempted from Wet Medisch-Wetenschappelijk Onderzoek (Law Medical Research) as a retrospective anonymous study.

2.2. DNA extraction

After the initial H&E stained slides, 4 (resection specimens) or 12 (biopsy specimens) additional slides were cut and mounted on slides for DNA extraction, followed by a final H&E stained section in order to check tumour availability. Guided by the H&E stained slides macrodissection was performed on the unstained slides discarding areas without tumour tissue. Genomic DNA was extracted with the QIAmp DNA FFPE Tissue kit (Qiagen, Venlo, NL) using the Qiacube automated method; concentration and purity of DNA was checked