Intra-tumoral genomic heterogeneity in rectal cancer: mutational status is dependent on preoperative biopsy depth and location.

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Abstract

Background Neoadjuvant therapy is indicated for patients with locally advanced rectal cancer, even though a significant number of patients show minimal or no response. Adequate response prediction before the start of neoadjuvant treatment might reduce unnecessary waiting periods and therapy related toxicity in non-responders. Genomic mutational status might provide a means to predict response to neoadjuvant therapy. However, it is unclear whether predictions based on genomic mutational status in single preoperative biopsies are reliable due to intra-tumoral heterogeneity. In this study we aim to investigate the reliability of genomic mutations found in single pre-operative biopsies by comparing these genomic mutations to 4 other locations within the same tumor using next generation sequencing.

Methods Rectal cancer patients undergoing primary resection, without neoadjuvant therapy, were included. Of all patients, one biopsy, two deep and two superficial samples were obtained and sequenced using a targeted next generation sequencing gene panel. Concordance between these 5 samples was assessed.

Results In this feasibility study we included 11 patients. In 7 out of 11 (64%) patients, all 5 samples showed concordant mutations. In 4 out of 11 patients (36%) disconcordant mutations were observed.

Conclusions Evaluation of genomic mutational status on a single pre-operative biopsy shows disconcordance in a substantial amount of patients.

Background

Locally advanced rectal cancer (LARC) patients are currently treated with neoadjuvant (chemo)radiotherapy followed by surgical resection (1). In clinical practice, the observed response to neoadjuvant therapy is heterogeneous. A pathological complete response (complete regression of tumor and/or pathological lymph nodes) is seen in 15-20% (up to 73% in some series) of patients, whereas in the vast majority of patients (54-75%) neoadjuvant therapy results in a partial response (2,3). Unfortunately, a subset of 10-50% of LARC patients receives futile neoadjuvant treatment when minimal or no response is observed (2,4). Currently, treatment is stratification as advised in national guidelines is based on clinical TNM stage, tumor distance to the mesorectal fascia and the presence of extramural vascular invasion (5). Response prediction based on parameters readily available before neoadjuvant treatment might provide a means to ensure patient-tailored treatment, and reduce unnecessary waiting periods and therapy related toxicity in non-responders.

Tumor associated immune response and intra-tumoral heterogeneity might be involved in causing therapeutic resistance of the tumor (6). Intra-tumoral genomic heterogeneity refers to the presence of genetically distinct sub clones within cancer lesions, and is developed by tumors in reaction to a diversity of microenvironmental factors including hypoxia, tissue stiffness, immune response and chronic inflammation or can be caused by the polyclonal origin of these tumors (7,8). Intra-tumoral genomic heterogeneity is particularly significant in colorectal cancer, and is attributed to the presence of both microsatellite- and chromosomal instability (9–11).
In previous studies the predictive value of (a combination of) clinical, pathological and radiological parameters in predicting response to (neoadjuvant) therapy has been assessed. Among these are several pathological expression indices (e.g. Ki-67, EGFR and P21) and genomic mutational status, as well as radiological parameters derived from MR and [¹⁸F]FDG-PET/CT imaging (e.g. standardized uptake value (SUV) and apparent diffusion coefficient (ADC))(12–20). Unfortunately, these studies so far have not resulted in clinically used prediction models.

The predictive value of genomic mutations in colorectal cancer has previously been investigated, concluding that KRAS, as well as RAS, BRAF and PIK3CA mutations, are predictive of tumor response to anti-EGFR therapy (17,18,21–25). Furthermore, a high degree of intra-tumoral genomic heterogeneity (as investigated by whole exome sequencing) has been associated with worse disease-free survival and was correlated with a higher rate of liver metastases (26). So far, no specific genomic mutations have been found to accurately predict response to neoadjuvant therapy in LARC patients (19).

A combination of genomic mutations might provide a means to predict response to neoadjuvant therapy in (locally advanced) rectal cancer patients before the start of neoadjuvant therapy. However, the reliability of next generation sequencing performed on routinely obtained single preoperative biopsies has yet to be established. Intra-tumoral heterogeneity has been shown to be significant in rectal tumors and their associated lymph nodes and metastases (27,28). Therefore, genomic mutations found in single preoperative biopsies might vary within individual patients, depending on the biopsy location and depth. It is therefore currently unclear whether predictions based on single preoperative biopsies are reliable to predict response to neoadjuvant therapy.

In this study we aim to investigate the reliability of genomic mutations found in a single preoperative biopsy by comparing these mutations to 4 other locations within the same tumor using next generation sequencing.

**Methods**

**Patients**

Rectal cancer patients from the Radboud University Medical Center, Nijmegen, the Netherlands and diagnosed between 2010 and 2012 with a biopsy confirmed rectal adenocarcinoma were retrospectively included in this study. To prevent any influence of neoadjuvant therapy on the results, patients
undergoing surgical resection of the primary tumor without neoadjuvant chemo- and/or radiotherapy were included.

Patient characteristics were obtained from medical records, including age, gender, clinical- and pathological characteristics. This project was conducted in accordance with the Declaration of Helsinki, and did not require approval of the local IRB according to local WMO regulations.

Tumor identification and DNA isolation

For each patient, five tissue samples were obtained from representative formalin-fixed paraffin-embedded (FFPE) tumor blocks containing material of 1 preoperative diagnostic biopsy, 2 superficial tumor tissue samples and 2 deep (central) tumor tissues samples of the resected specimen. Optimal FFPE blocks (with adequate tumor cellularity of ≥20% from full samples, and >10% in biopsy samples) for smMIP analysis were identified and marked by an expert pathologist (I.N.) on representative hematoxylin and eosin (H&E) stained slides. When inadequate tumor cell percentages were obtained, this was determined as insufficient reading depth and the sample was not further analysed. To obtain sufficient genomic DNA, marked tumor areas were cut out from 10 sequential (non-stained) slides (each 6 µm thick). DNA was isolated at 56 °C for 1 hour using TET-lysis buffer with 5% Chelex-100 (Bio-Rad, Hercules, USA) and 400µg proteinase K (Qiagen, Valencia, USA), followed by inactivation at 95°C during 10 minutes (29). The DNA concentration was determined using the Qubit High Sensitivity Kit (Invitrogen, Carlsbad, USA) per manufacturer’s protocol.

smMIP sequencing

A panel of 911 smMIPs was used to detect variants in 31 cancer-related genes, as displayed in Table 1. To provide gender control, smMIPs targeting AMELX and AMELY were included. The smMIP sequencing protocol has previously been clinically validated and used in the Radboud University Medical Center (29). One hundred nanogram of isolated DNA was included per sample. After sample preparation, manual library preparation was performed (29). The purified libraries were diluted. Sequencing was performed using the NextSeq500 (Illumina, San Diego, USA) per manufacturer's protocol (300 cycles High Output sequencing Kit, Illumina, San Diego, USA), resulting in 2 x 150 bp paired end reads.

Sequence data analysis

Sequence data was generated from the NextSeq500, after which Bcl to FASTQ conversion and demultiplexing of barcoded reads was automatically performed. Sequence Pilot software (JSI Medical
Systems GmbH, Ettenheim, Germany) was used for generating consensus reads and variant identification, with settings as previously described (29). Variants found in samples passing gender control and exceeding an average minimum reading depth of 180 were automatically filtered with an in-house Python script, as depicted in Figure 1. This threshold excludes, with a certainty of >95%, the presence of a mutation at minimally 10% mutant allele frequency within covered regions. As SOX9 and SEC63 have many pseudogenes resulting in uncertainty about found mutations, we have excluded these from these analysis. Due to a technical sequencing artifact (in all samples), PTEN mutation c.407G>A was excluded from the analysis.

**Statistical analysis**

Statistical analysis was performed using SPSS version 23 (SPSS, Inc., Chicago, USA). Numerical data is presented as mean (standard deviation) or median (interquartile range) based on distribution. Categorical data is presented as frequencies and percentages. In order to quantify tumor heterogeneity, differences in mutational status between biopsy, deep and superficial tumor samples were analyzed by calculating the percentages of concordance and disconcordance. Concordance was defined as all five samples (1 biopsy, 2 deep samples, and 2 superficial samples) showing identical (or no) mutations. Disconcordance was defined as ≥1 mutation(s) in either of the 5 samples, which was not found in (one of) the other samples. For all tests performed, P<0.05 was considered statistically significant.

**Results**

**Patients**

Data and tissue of 11 patients were included in this study. Patients were on average 72 ± 27.4 years old, and consisted of 6 men and 5 females. Of these, 9 had a pT3 tumor and 2 a pT4 tumor. All patients were treated with immediate resection of the rectal tumor, without prior chemo- and/or radiotherapy. The rectal tumor was on average located 57.8 ± 46.3 mm from the anal verge, and measured 53.5 ± 21.6 mm in diameter. Detailed clinicopathological features are summarized in Table 2.

**Mutation concordance**

Thirty-three genomic mutations were found in the following 8 genes: APC (9/11), BRAF (1/11), FBXW7 (2/11), KRAS (7/11), PIK3CA (1/11), PTEN (6/11), SMAD4 (1/11) and TP53 (6/11). Insufficient (partial) reading depth was found in biopsy samples of 3 patients (patient 5, 8 and 9).

In 7 out of 11 (64%) patients, all 5 samples showed concordant mutations. In 4 out of 11 patients (36%) a disconcordance in mutations was observed within the 5 samples.
In patient 2 a disconcordance in *KRAS* (2 different mutations), *SMAD4* and *TP53* mutations was found between the superficial sample and the biopsy as well as both deep samples. Patient 4 showed disconcordance as the *TP53* mutation was only found in the biopsy and one of two superficial samples. Patient 5 showed disconcordance as the *APC* mutation was only found in the superficial samples compared to the deep samples (biopsy results were not available). In patient 8 disconcordance was found as different *TP53* mutations were found in the biopsy compared to the deep and superficial samples. These results are depicted in Figure 2 and 3.

**Discussion**

Response to neoadjuvant therapy is heterogeneous in LARC patients (2,4). Currently, neoadjuvant therapy is indicated for all LARC patients, even though a significant subset of patients is therapy resistant. Adequate stratification based on parameters available before treatment might enable better application of neoadjuvant therapy. Genomic mutational status is one of these parameters, and is routinely obtained in the diagnostic work up.

As this and previous research indicates, the use of a single predictive biomarker often underestimates the complex mechanisms involved in response prediction. In this study, genomic mutations in pre-operative biopsies were compared to 4 other locations within the same tumor using next generations sequencing. In 36% of the patients, evaluation of genomic mutational status on a single pre-operative biopsy has shown disconcordance between the various samples. This illustrates the genomic variability in rectal cancer and explains the difficulties in obtaining reliable biomarkers for response prediction. These results are in line with previous evidence supporting the presence of intra-tumoral genomic heterogeneity in a considerable proportion of rectal cancers (30). Three previous studies have compared genomic mutations in up to 3 intra tumoral locations. Hardiman *et al.* reported up to 10 coding variants uniquely corresponding to one of 3 of the tumor locations in their study of 6 patients (30). In the study of Bettoni *et al.*, only 27% of the observed mutations corresponded to all three samples of a single rectal adenocarcinoma in one patient (31). On the other hand, Dijkstra *et al.* reported no differences in mutational status between deep and superficial colorectal cancer tissue in 30 patients (32).

This study has several limitations. First of all, the small sample size and limited targeted next generation sequencing panel might influence the interpretation of results. The number of disconcordant cases might actually be higher, as this targeted gene panel only provides information on a selected number of mutations. Furthermore, there is no 100% certainty the found mutations were not germ-line mutations, however considering the observed allelic frequency this is very unlikely. Also, in biopsy material from 3 patients, insufficient reading depth was achieved. This indicates that estimating the tumor cell percentage on the (biopsy) FFPE blocks is challenging, and may lead to insufficient percentages of tumor cells with insufficient reading depth as a consequence. Furthermore, since the main aim of this study was
to determine the reliability of response prediction based on a single preoperative biopsy rather than define the degree of intra-tumoral genomic heterogeneity, the influence on our results would be limited. To increase the reliability of the biopsy analysis, the use of multiple and possibly even deeper/larger preoperative biopsies might provide a better representation of intra-tumoral heterogeneity, but might also increase the risk of procedure related complications. A second possibility might be the application of whole exome sequencing or larger targeted gene panels (such as the TSO500, Illumina, San Diego, USA), as this possibly provides a more elaborate analysis of genomic mutations, as compared to next generation sequencing using a limited targeted gene panel. Using these techniques, the mutant-allele heterogeneity (MATH) score was developed to quantitatively assess the spread of allele frequencies, and has been correlated to response (19,33). However, as sampling errors are innate to the biopsy technique, parameters derived from full tumor imaging might be preferable to incorporate characteristics of all genetic sub clones present in these cancers.

In the future, pretreatment biopsies may be used to predict response to therapy and might provide an additional tool for patient stratification and the application of neoadjuvant therapy. As our results demonstrate a degree of disconcordance between biopsies taken from various locations, predicting algorithms should include various clinical, radiological and pathological parameters to overcome the complexity of tumor heterogeneity.

**Conclusion**

In conclusion, assessment of overall tumor heterogeneity on a single pre-operative biopsy shows disconcordance and the use of it to predict response to neoadjuvant therapy is warranted. As we aim for tailored treatment and optimized patient outcome, predictive algorithms including multiple features will have to be developed to further stratify patients to optimal treatment regimens.

**Abbreviations**

| Abbreviation | Description                        |
|--------------|-----------------------------------|
| LARC         | Locally advanced rectal cancer    |
| SUV          | Standardized uptake value         |
| ADC          | Apparent diffusion coefficient    |
| FFPE         | Formalin fixed paraffin embedded  |
| H&E          | Hematoxylin and eosin             |
| MATH         | Mutant allele tumor heterogeneity |

**Declarations**
Ethics approval: This study was conducted in accordance with the declaration of Helsinki, and did not require approval of the local IRB according to local WMO regulations.

Consent for publication: Not applicable.

Availability of data and materials: The datasets generate and analyzed in this study are not publicly available for the reason of protecting patients’ privacy, but are available from the corresponding authors on reasonable request.

Competing interests: The authors of this manuscript have nothing to disclose.

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Author contributions: FV, MV, IN and DH were in charge of the study design, data review and manuscript preparation. CW and SV contributed to data analysis and figure preparation. CV and AV contributed to the manuscript preparation. All authors read and approved the final manuscript.

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Tables

**Table 1. Overview of Regions Targeted by the Transcan smMIP Panel.**
| Gene     | Transcript ID (RefSeq) | Transcript ID (Ensembl) | Exon number | Targeted regions                                         | Positions analyzed for variants |
|----------|------------------------|-------------------------|-------------|----------------------------------------------------------|-------------------------------|
| ACVR1B   | ENST000002 57963       | NM_004302               | 02          | Activin types I and II receptor domain                   | c.92-5 to c.331+5             |
|          |                       |                         |             | Transforming growth factor beta type I GS-motif          | c.556 to c.1518+5             |
|          |                       |                         |             |                                                          |                               |
| ACVR2A   | ENST000002 41416       | NM_001616               | 06-11       | Protein kinase domain                                     | c.673-5 to c.1542+5           |
| AMER1    | ENST000003 30258       | NM_152424               | 02          | WTX Protein                                             | c.639 to c.1629               |
| APC      | ENST000002 57430       | NM_000038               | 01-16       | Whole gene                                               | c.8532+5 to c.3397            |
| ARID1A   | ENST000003 24856       | NM_006015               | 11-12       | ARID DNA-binding domain                                  |                               |
|          |                       |                         |             | SWI/SNF-like complex subunit BAF250/Osa                 |                               |
|          |                       |                         |             | Immunglobulin C1-set domain                              | c.68-5 to c.346+5             |
| B2M      | ENST000005 58401       | NM_004048               | 02          |                                                          |                               |
| BRAF     | ENST000002 88602       | NM_004333               | 15          | Codon D594-K601                                          | c.1742-5 to 1860+5           |
| CASP5    | ENST000003 93141       | NM_004347               | 02-03       | CARD domain                                             | c.8 to 433+5                  |
| CASP8    | ENST000003 58485 25    | NM_0010801              | 07-09       | Caspase domain                                           | c.838 to 1617+5               |
| CTNNB1   | ENST000003 49496       | NM_001904               | 03          | Codon D32-S45                                           | c.36 to c.163                 |
| EGFR     | ENST000002 75493       | NM_005228               | 12          | Receptor L domain                                        | c.1082-5 to c.1185+5         |
|          |                       |                         |             | Protein tyrosine kinase                                  | c.1391 to c.1498+5           |
| ERBB2    | ENST000002 69571       | NM_004448               | 18-24       | Protein tyrosine kinase                                  | c.2062-5 to 2625+5           |
| FBXW7    | ENST000002 81708       | NM_033632               | 07-12       | WD domain, G-beta repeat                                 |                               |
| GNAS     | ENST000003 71085       | NM_000516               | 08-09       | Codon R201 and Q227                                      | c.1035 to c.2124+5           |
| IDH2     | ENST000003 30062       | NM_002168               | 04          | Codon R140 and R172                                      |                               |
| KRAS     | ENST000003 11936       | NM_004985               | 02          | Codon G12, and G13                                       | c.1-5 to c.111+5             |
|          |                       |                         |             | Codon A59 and Q61                                       | c.112-5 to c.232             |
|          |                       |                         |             | Codon K117 and A146                                      | c.385 to c.402               |
|          |                       |                         |             |                                                          | c.402 to c.450+5             |

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| Gene  | Ensembl ID | Entrez ID | Function                                                                 | Protein Region | Mutation Details                             |
|-------|------------|-----------|---------------------------------------------------------------------------|----------------|-----------------------------------------------|
| MET   | ENST000003 | NM_0011275| Protein tyrosine kinase                                                   | c.3140         | to c.4227+5                                   |
| NRAS  | ENST000003 | NM_002524 | Codon G12 and G13, Codon A59 and Q61, Codon E542 to Q546, Codon M1043 to G1049 | c.1-5 to c.99  | to c.272                                      |
| PIK3CA| ENST000002 | NM_006218 | DNA-directed DNA polymerase, family B, exonuclease domain                  | c.1-5 to c.99  | to c.1557 to c.1664+5                         |
| POLE  | ENST000003 | NM_006231 | DNA-directed DNA polymerase, family B, exonuclease domain                  | c.1-5 to c.99  | to c.3041 to c.3207+5                         |
| PTEN  | ENST000003 | NM_000314 | Dual specificity phosphatase, catalytic domain, C2 domain of PTEN tumor-suppressor protein | c.310          | to c.1026+5                                   |
| RNF43 | ENST000004 | NM_017763 | Whole CDS                                                                 | c.1-5 to c.99  | to c.2352+5                                   |
| SMAD2 | ENST000002 | NM_005901 | Whole CDS                                                                 | c.1-5 to c.99  | to c.1404+5                                   |
| SMAD4 | ENST000003 | NM_005359 | MH1 domain                                                                | c.1-5 to c.99  | to c.250-5                                    |
| SMARCA2| ENST000003| NM_003070 | SNF2-related, N-terminal domain                                           | c.1-5 to c.99  | to c.454+5                                    |
| SMARCA4| ENST000004| NM_0011288| SNF2-related, N-terminal domain                                           | c.1-5 to c.99  | to c.956-5                                    |
| SMARCA5| ENST000004| NM_0011288| SNF2-related, N-terminal domain                                           | c.1-5 to c.99  | to c.1659+5                                   |
| SMARCB1| ENST000002| NM_003073 | SNF5/SMARC B1/INI1                                                         | c.1-5 to c.99  | to c.2185-5                                   |
| SOX9  | ENST000002 | NM_000346 | Whole CDS                                                                 | c.1-5 to c.99  | to c.3078+5                                   |
| TCF7L2| ENST000003 | NM_030756 | CTNNB1 binding, N-terminal                                               | c.1-5 to c.99  | to c.3168+5                                   |
| TGFBR2| ENST000003 | NM_0010248| High mobility group box domain                                            | c.1-5 to c.99  | to c.933-5                                    |
| TP53  | ENST000002 | NM_000546 | P53 DNA-binding domain                                                     | c.1-5 to c.99  | to c.919+5                                    |
| Variables                              | N=11 |
|---------------------------------------|------|
| **Age (years)**                       | Mean (SD) 72.2 (27.4) |
| **Gender**                            | Male 6 (55%) |
|                                       | Female 5 (45%) |
| **pT**                                | 3 9 (82%) |
|                                       | 4 2 (18%) |
| **pN**                                | 0 6 (55%) |
|                                       | 1 3 (27%) |
|                                       | 2 2 (18%) |
| **EMVI**                              | Yes 4 (36%) |
|                                       | No 6 (55%) |
|                                       | Missing 1 (9%) |
| **Differentiation**                   | Good 9 (82%) |
|                                       | Bad 1 (9%) |
|                                       | Missing 1 (9%) |
| **Distance to CRM (mm)**              | Mean (SD) 14.1 (7.7) |
| **Diameter tumor (mm)**               | Mean (SD) 53.5 (21.6) |
| **Total number of lymph nodes**       | Median (IQR) 15 (12-19) |
| **Number of tumor positive lymph nodes** | Median (IQR) 0 (0-3) |
| **Distance from anal verge (mm)**     | Mean (SD) 57.8 (46.3) |

Abbreviations: SD, standard deviation; pT, clinical tumor stage; pN, clinical nodal stage; EMVI, extramural vascular invasion; CRM, circumferential resection margin; IQR, interquartile range.

**Figures**
Remove non-coding variants (except for frameshift mutations)

Remove variants in homopolymeric regions (minimum size of 6N repeats)

Remove single nucleotide polymorphisms with a frequency of > 0.01 in gnomAD

Remove synonymous variants

Remove UTR variants

Remove PTEN, SOX9 and SEC63 variants suggestive for pseudogenes

Remove variants with a > 3 fold Fw/Rev read imbalance
Figure 1

Flowchart of smMIP analysis data filtering. Overview of data filtering before smMIP data analysis.

Figure 2

Graphical display of mutations in all samples. Representation of mutations found in deep, superficial and biopsy samples.

1. APC
2. BRAF
3. FBXW7
4. KRAS
5. PIK3CA
6. PTEN
7. SMAD4
8. TP53
Figure 3

Overview of specific mutations. Overview of specific mutations found in all samples.